Remarks

Applicant has carefully considered this Application in connection with the Examiner's Action, and respectfully requests reconsideration of this Application in view of the foregoing amendment, and the following remarks.

Applicant would like to thank the Examiner for withdrawing the rejection of Claim 4 under 35 U.S.C. § 112, second paragraph.

Claim 1 is amended to more clearly define the subject matter of Applicant's invention. Support for the amendment is found in the specification and claims as originally filed. No new matter is added. Claims 1 and 3-5 are presently pending in the Application.

Applicant herewith submits copies of scientific publications in support of remarks made in response to the rejections, which are outlined below. Specifically, the scientific publications include:

1. Schauber, *et al.*, Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel disease, Europ. J. of Gastroenterology & Hepatology, Vol. 18, No. 6 (2006).

2. Hata, *et al.*, Abstract 271, J. of Invest. Derm., Vol. 127 (2007).

3. Howell, *et al.*, Cathelicidin deficiency predisposes to eczema herpeticum, , J. Allergy & Clin. Immunol., Vol. 117, No. 4 (2006)

4. Zhang, *et al.*, Cationic antimicrobial peptides – an update, Expert Opin. Invest. Drugs, Vol. 13, No. 2 (2204).

5. Büchau *et al,* Abstract 777, J. of Invest. Derm., Vol. 127 (2007).

6. Bornhovd, *et al.*, Macrolactam immunomodulators for topical treatment of inflammatory skin diseases, J. Am. Acad. Dermatol., Vol. 45, No. 5 (2001).

7. Gupta, *et al.*, Pimecrolimus: a review, Europ. J. of Derm. & Venerol., Vol. 17 (2003).

I. Rejection under 35 U.S.C. § 112, First Paragraph

Claim 3 stands rejected under 35 U.S.C. § 112, First Paragraph for enablement. The Examiner asserts that the specification does not enable any person skilled in the art to which it pertains to make or use the invention. More specifically, the Examiner contends that while the specification supports enablement for synergistic effects as it pertains to inhibition of T-cell proliferation, it does not reasonably provide enablement

for a claim that reads on any synergistic effect. The Examiner goes on to state that no facts or examples have been presented to show that the compounds act synergistically with respect to the disease. Applicant respectfully traverses the rejection for the reasons discussed below.

Applicant need not have actually reduced the invention to practice prior to filing in order to satisfy the enablement requirement under 35 U.S.C. §112, first paragraph. MPEP §2164.02 (citing *Gould v. Quigg*, 822 F.2d 1074 (Fed. Cir. 1987)). Indeed, the invention need not contain a single example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation (*In re Borkowski*, 422 F.2d 904, 164 U.S.P.Q. 642 (CCPA 1970)), and "representative samples are not required by the statute and are not an end in themselves" (*In re Robins*, 429 F.2d 452, 456-57, 166 U.S.P.Q. 552, 555 (C.C.P.A. 1970)). Thus, 35 U.S.C. § 112, first paragraph, enablement does not require any working examples.

The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. MPEP §2164.01 (citing *In re Angstadt*, 537 F.2d 498, 504 (C.C.P.A. 1976)). The fact that experimentation may be complex does not necessarily make it undue if the art typically engages in such experimentation. *Id.* Further, the specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. MPEP §2164.05(a) (citing *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991)). Therefore, enablement does not require a working example and experimentation is allowed so long as it is not undue.

Claim 3 is enabled under 35 U.S.C. §112, first paragraph because the specification as filed amply supports claims reciting a method comprising administering to a patient in need thereof of a synergistically effective amount of a pharmaceutical composition comprising pimecrolimus in combination with calcipotriol or tacalcitol. Applicant does not teach "any synergistic effect", but rather, expressly recites a synergistically-effective amount of pimecrolimus in combination with calcipotriol or tacalcitol to treat acne, psoriasis or IBD.

Applicant directs the Examiner to paragraph 41 of the specification, and to Berenbaum and EPO427680 and EPO683156, which are cited therein at paragraph 42,

for representative conventional methods for calculating and verifying synergetic effects of pharmaceutical compositions.

Moreover, Applicant directs the Examiner to the specification at page 8, in which an example is provided for the use of pimecrolimus in combination with calcipotriol, including suitable unit dosages for oral administration. Specifically, Applicant teaches "synergistically effective amounts of 33-epichloro-33-desoxyascomycin and calcipotriol on oral administration for use in treating atopic dermatitis, acne or psoriasis, or of IBD, in larger animals, e.g. man, are amounts of 33-epichloro-33-desoxyascomycin of up to 2 mg/kg/day, e.g. from about 0.01 mg/kg/day to about 2 mg/kg/day, preferably about 0.5 mg/kg/day, in combination or co-administration with amounts of calcipotriol from about 0.25 mg/kg/day to about 50 mg/kg/day, preferably 2.5 mg/kg/day, in a synergistic ratio". (See specification, page 8, lines 1 – 19.) Synergistic ratios, by weight, include "1000:1 to about 1:10, most preferably from 200:1 to about 20:1. " (See specification, page 7, lines 17 – 20.)

It must also be noted that calciferols share the same mode of action, although they differ in physical and chemical properties. Accordingly, results obtained with pimecrolimus and vitamins D3, one example of a calciferol, are expected to be the same when pimecrolimus is combined with another calciferol.

Applicant further submits that specific post-published data, as presented herewith in the form of research publications, is sufficient to show the link between the effect of the composition and the diseases as claimed by Applicant. The following scientific publications are provided in order to support the surprising existence of a link between the induction of cathelicidin mRNA and the diseases of the skin, like IBD, atopic dermatitis and acne. Specifically, Schauber *et al.* describes that cathelicidin expression is altered in IBD. Hata *et al.* (Abstract) discloses that a down-regulation of cathelicidin mRNA increases the susceptibility of vaccinia virus infection in atopic dermatitis, and that cathelicidin is deficient in atopic dermatitis skin. Howell *et al.* describes that AMPs are decreased in the skin of patients with atopic dermatitis, and that cathelicidin is such an AMP. (See Howell, page 863 and 839.) Zhang *et al.* describes the use of cationic AMPs as modulators in the treatment of acne. (See Zhang, pages 101 and 102, left columns.)

With respect to the surprising therapeutic effect seen by the combination of pimecrolimus and a calciferol, the scientific data presented herewith, specifically shown in Büchau *et al.*, provide support that the combination of pimecrolimus and vitamin D3 (cholecalciferol) increases the NHEK expression of cathelicidin AMP mRNA by 205% compared to pimecrolimus alone. Thus, the scientific publications show the surprising existence of a link between the induction of cathelicidin mRNA and the diseases of the skin, like IBD, atopic dermatitis, and acne. Therefore, a compound modifying the regulation of cathelicidin mRNA is expected to have an effect on the above diseases.

Armed with the teachings of Applicant's invention and what was known in the art, the practioner would not need to engage in any undue experimentation to practice the invention commensurate with the scope of Claim 3, and Claim 3 is therefore enabled under 35 U.S.C. § 112.

As such, Applicant respectfully requests that the Examiner reconsider and withdraw the rejection of Claim 3 under 35 U.S.C. § 112, First Paragraph.

II. Rejection under 35 U.S.C. § 103(a)

Claims 1, 3-5 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Van Etten et al. in view of WO 09/18468; Nghiem et al.; Paul et al.; Baumann et al. (U.S. Patent No. 5,912,238); Van De Kerkhof et al.; and Koo et al. (U.S. Pub. No. 2004/0202706). For the reasons stated below, Applicant respectfully traverses the rejection.

A. <u>The prior art does not disclose pimecrolimus in combination with calcipotriol or tacalcitol, together with at least one pharmaceutically acceptable diluent or carrier:</u>

To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir.1991).

The Examiner states that the prior art discloses the combination of a macrolide T-cell immunomodulator and a calciferol with a pharmaceutical carrier. The difference between the prior art and the claimed invention is that the prior art does not expressly disclose the combination of pimecrolimus and calcipotriol or tacalcitol, a method of treatment of a dermatological disease or inflammatory bowel disease with a macrolide T-cell immunomodulator and a calciferol in synergistically effective amounts.

The Examiner, rather, states that the prior art suggests such, because the prior art discloses the combination of rapamycin and 1,25(OH)2D3 for suppressing T-cell proliferation.

The Examiner cites Van Etten for teaching a combination of rapamycin or tacrolimus (FK506) and the vitamin D analog $1,25(OH)_2D_3$. The Examiner also cites WO 98/18468 for the combination of rapamycin and $1,25(OH)_2D_3$ for the treatment of diseases of the immune system. A third reference, Nghiem is cited for teaching pimecrolimus and tacrolimus as structurally similar macrolide immunosuppressant. A fourth reference, Paul, is cited for teaching ascomycin derivatives as a novel class of anti-inflammatory macrolides. A fifth reference, Van de Kerkhof, is cited as teaching calcipotriol and tacalcitol as less hypercalcemic than the vitamin D analog $1,25(OH)_2D_3$. Bauman is cited by the Examiner for teaching pimecrolimus in the treatment of autoimmune and hyper-proliferative skin diseases.

Applicant respectfully asserts that the references cited by the Examiner, when combined, fail to teach the limitations of Claims 1 and 3, which recite the use of a synergistic amount of pimecrolimus in combination with calcipotriol or tacalcitol for the treatment of dermatological diseases and inflammatory bowel disease (IBD). As such, the references do not teach each and every element of Applicant's claimed invention.

Moreover, Applicant believes that the Examiner has incorrectly described the teachings of the references. In other words, Applicant respectfully asserts that the references do not say what the Examiner says that they do. Specifically, Van Etten presents as the goal of its study whether synergism could be observed with immunosuppressants other than rapamycin, namely mycophenolate mofetil, leflunomide and the methylxanthine A802715, or with analogs of $1,25(OH)_2D_3$. Variability in synergism for the different combinations were noted – synergism was not consistent - and was dependant on several factors, such as the kind of immunomodulator used, the

kind of vitamin D analog used, dosages used, etc. Moreover, Van Etten does not teach nor mention pimecrolimus and even teaches away from the combination of immunosuppressants with vitamin D analogs because of the undesired calcemic effects seen with $1,25(OH)_2D_3$.

Nghiem is cited as teaching tacrolimus, however, this does not cure the defects of Van Etten because it does not suggest tacrolimus in combination with a vitamin D derivative. Nghiem is solely directed at a topical treatment for atopic dermatitis and there is no teaching or suggestion to applications beyond such treatment. It should also be noted that tacrolimus is not an ascomycin derivative, as is pimecrolimus, thus it would not motivate one skilled in the art to merely substitute pimecrolimus for tacrolimus as the Examiner seems to suggest.

While WO 09/18468 teaches the use of vitamin D derivatives, it is solely directed to rapamycin and calcitriol and does not teach calcipotriol or tacalcitol. Rapamycin is not a calcineurin inhibitor, as is pimecrolimus, thus there would be no basis for modifying the reference in a manner suggested by the Examiner.

Van de Kerkhof teaches tacalcitol ointment in the treatment of psoriasis. There is no discussion of combining tacalcitol with other compounds and treatment is solely directed to psoriasis. Nothing in Van de Kerkhof suggests applications to other diseases.

The Examiner cites Paul as teaching ascomycin derivatives as a novel class of anti-inflammatory macrolactams. Paul teaches tacrolimus for the treatment of psoriasis and discloses that "tacrolimus is not an ascomycin derivative". (See Paul, page 70.)

Thus, at the time of the invention, not only was there simply no disclosed use of pimecrolimus in combination with calcipotriol or tacalcitol for the treatment of dermatological diseases and IBD, there was no suggestion or motivation in the art to arrive at Applicant's invention. Moreover, Applicant respectfully asserts that a person of ordinary skill in the art would not be motivated or suggested to combine the references as suggested by the Examiner.

For instance, a person of ordinary skill would know that while rapamycin and tacrolimus are related substances, they are isolated from different species of streptomyces and have different modes of action. For example, rapamycin is not a calcineurin inhibitor – as is pimecrolimus – and tacrolimus is not an ascomycin

derivative – as is pimecrolimus. Thus, a person of skill in the art would not consider all macrolides as having the same functions and activities, in particular since this group is also a chemically diverse one. Furthermore, a synergistic effect of a combination of the macrolide with a vitamin D derivative cannot be expected for all combinations of macrolides.

The Examiner states that calcipotriol and tacalcitol are disclosed to be effective in treating psoriasis; however, all teachings are for the vitamin D analogs alone – in topical ointments – to treat psoriasis. Nowhere is there a teaching or suggestion of oral dosage forms for the treatment of inflammatory bowel disease, or a suggestion that their efficacy would be improved by co-administration with another therapeutic. Moreover, the teachings of the prior art underscore what a diverse group of compounds macrolides comprise and therefore, one skilled in the art would know that one macrolide cannot simply be substituted for another, as suggested by the Examiner.

No use of a synergistically effective amount of pimecrolimus in combination with a calcipotriol or tacalcitol for the treatment of dermatological diseases and IBD, as expressly required by Applicant's Claim 3 is taught or suggested by the references cited by the Examiner. Thus, Applicant asserts that alone, or in combination, the art cited by the Examiner fails to teach or suggest each and every one of the expressly required elements of Applicant's claimed invention. Accordingly, Applicant respectfully requests that the rejection under 35 U.S.C. §103 be reconsidered and withdrawn.

B. <u>One of ordinary skill in the art would not be motivated to combine the references as suggested by the Examiner because of the difference in therapeutic effectiveness of pimecrolimus, tacrolimus and rapamycin, as well as in their structure-related limitations of formulation</u>:

The Examiner asserts that one of ordinary skill in the art would have been motivated to combine and modify the prior art with the expectation that due to similar structure and effect on dermatological disease, that pimecrolimus would be a suitable substitute for rapamycin or FK506 and act synergistically in the inhibition of T-cell proliferation in combination with 1,25(OH)2D3 and analogues thereof.

On the contrary, the established differences between the macrolide compounds preclude simply substituting one macrolide for another as suggested by the Examiner. Submitted herewith and attached hereto, are several scientific publications highlighting the understanding of macrolactam immunomodulators, as they were, at the time of the

invention. For instance, a clinic review of macrolactam immunomodulators for topical treatment of inflammatory skin diseases in the Journal of the American Academy of Dermatology discusses the differences between pimecrolimus, tacrolimus and rapamycin (Bornhovd *et al*). A review of pimecrolimus in the European Journal of Dermatology and Venereology discusses its mechanism of action and use in treatments (Gupta *et al*).

With regard to pimecrolimus and tacrolimus: "they differ in therapeutic effectiveness as well as in their structure-related limitations of formulation." (Bornhovd, page 738)

With regard to rapamycin (also called sirolimus): "rapamycin binds to macrophilin-12 and other immunophilins. The biological effects of the rapamycin-macrophilin-12 complex are different from those of other drug-macrophilin complexes discussed (pimecrolimus). The target structures of the rapamycin-macrophilin-12 complex are a group of proteins named mammalian targets of rapamycin, also known as FK-BP-rapamycin-associated protein." And further, "rapamycin affects the cell cycle of the activated cell (G1 – S phase), whereas pimecrolimus blocks the cell cycle in an earlier phase (G0)." (Bornhovd, page 738)

Moreover, the immunosuppressive qualities of topical rapamycin/sirolimus application have been studied in different animal models with contradictory results. Topical application of 1.2% and 2.0% rapamycin/sirolimus ointment were ineffective in a guinea pig contact dermatitis model – although suppression of keratinocyte proliferation was observed. (Bornhovd, page 739)

On the contrary, pimecrolimus is an immunophilin ligand which binds macrophilin-12 and inhibits protein phosphatase calcineurin. (Gupta, Abstract) Calcineurin is a calcium-calmodulin-dependent protein phophatase that regulates the translocation of cytosolic components of nuclear factors, which in turn regulate the promoter activity of several mediators during mRNA transcription. (Gupta, page 494) Pimecrolimus also targets mast cells. (Gupta, page 494) Moreover, pimecrolimus posses two different chemical group attachments versus tacrolimus; pimecrolimus is 20 times more lipophilic than tacrolimus. A higher lipophilicity allows pimecrolimus to have a higher affinity to the skin; as a result, it has a lower permeation potential through the skin, with a skin-selective pharmacological profile. (Gupta, page 501)

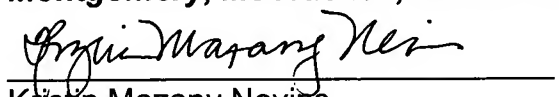
In conclusion, and supported by the attached scientific articles, rapamycin and pimecrolimus operate biologically by absolutely unique pathways; rapamycin binds FKBP-rapamycin associated protein and inhibits TOR pathway, whereas pimecrolimus inhibits calcineurin, and cannot simply be substituted for another as the Examiner suggests to arrive at Applicant's Claims 1, 3 – 5.

No use of a synergistically effective amount of pimecrolimus in combination with a calcipotriol or tacalcitol for the treatment of dermatological diseases and IBD, as required by Applicant's Claim 3 is taught or suggested by the references cited by the Examiner. As such, Applicant respectfully requests that the rejection under 35 U.S.C. §103 be reconsidered and withdrawn.

III. Conclusion

In view of the foregoing, Claims 1 and 3-5 are in condition for allowance, and Applicant earnestly solicits a Notice of Allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this Application, the Examiner is invited to telephone the undersigned at the number provided.
Prompt and favorable consideration to this Amendment and Reply is respectfully requested.

Respectfully submitted,
Montgomery, McCracken, Walker & Rhoads, LLP



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Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel diseases

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Background Inflammatory bowel diseases (IBDs) are characterized by a breakdown of colon epithelial barrier function. Antimicrobial peptides like cathelicidins are molecules of the innate immune system located at epithelial surfaces. Cathelicidins influence microbial growth and inflammation and may play a role in IBD. In this study, the expression of human cathelicidin hCAP18/LL-37 was investigated in the intestinal mucosa from patients suffering from ulcerative colitis or Crohn's disease.

Methods Biopsy material from colon and ileal mucosa of a total of 89 patients (34 with Crohn's disease, 27 with ulcerative colitis, 28 control patients) was evaluated for cathelicidin expression by real-time reverse-transcriptase polymerase chain reaction and immunohistochemistry. Colon epithelial cells were stimulated *in vitro* with various cytokines to evaluate mechanisms that influence cathelicidin production.

Results Cathelicidin expression was significantly increased in inflamed and non-inflamed colon mucosa from ulcerative colitis patients compared to non-inflamed control mucosa. In patients with Crohn's disease cathelicidin expression was not changed in inflamed or non-inflamed colon or ileal mucosa independent of NOD2 status. Biopsies evaluated by immunohistochemistry showed epithelial cathelicidin expression in the upper crypt that was diffuse in controls and only basal in IBD patients. Inflammation mediators, alone or in combination with the known cathelicidin inducer butyrate, had no effect on cathelicidin expression in cultured colon cells.

Conclusions In IBD the colonic expression of human cathelicidin is altered: cathelicidin expression is increased in inflamed and non-inflamed mucosa in patients suffering from ulcerative colitis but not in Crohn's disease. This deficiency may further compromise the antimicrobial barrier in Crohn's disease. *Eur J Gastroenterol Hepatol* 18:615–621 © 2006 Lippincott Williams & Wilkins.

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Keywords: antimicrobial peptides, innate immunity, cathelicidin LL-37, inflammatory bowel disease, Crohn's disease, ulcerative colitis

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Introduction

A breakdown of intestinal barrier function has been observed in patients suffering from inflammatory bowel diseases (IBDs) [1,2]. Bacterial antigens may cross the epithelial cell lining and evoke an inflammatory response resulting in the clinical picture of ulcerative colitis or Crohn's disease. Several findings argue for a central role of the intestinal flora in the pathogenesis of IBD. For example, in animal models germ-free conditions prevent mucosal inflammation [3], T-cell responses in IBD patients are directed against the autologous flora [4], in Crohn's mucosa adherent *Escherichia coli* strains have been found [5] and, finally, antibiotics are effective in ameliorating symptoms in IBD patients [2].

Human colon epithelium is armed with an array of effector molecules including antimicrobial peptides in order to maintain an active barrier against the intestinal flora [6–8]. These peptides can be considered as endogenous antibiotics and are widespread in nature as immediate defence effectors [9]. Human antimicrobial peptides include defensins and cathelicidins. These gene families differ significantly in gene regulation, peptide structure and antimicrobial spectrum [10]. The cathelicidins constitute a family of precursor proteins with a well-conserved cathelin pro-region, followed by a highly variable C-terminal antimicrobial domain [11]. After cleavage from the cathelin pro-region the only human cathelicidin precursor protein hCAP18 gives rise to an active peptide named LL-37, a 37-residue mature cationic and amphipathic peptide [12–14]. Cathelicidin is present in neutrophils and lymphocytes [13,15]. In addition, cathelicidin is synthesized by lung epithelium [16] and various squamous epithelia [17]. Recently, it was

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also shown that differentiated cells of human distal small intestine and colon express cathelicidin [18]. Cathelicidin is also expressed by gastric epithelial cells and contributes to host mucosal defence against *Helicobacter pylori* [19]. Besides their antimicrobial activity, cathelicidins such as LL-37 have a variety of other functions such as chemoattraction of immune cells, release of histamine from mast cells, modulation of inflammation, or induction of angiogenesis [20].

There is evidence that expression of antimicrobial peptides is altered in IBD: human β-defensin 2 (HBD-2) and HBD-3 is induced in colon epithelium in ulcerative colitis patients. In contrast, in Crohn's disease patients no induction of these inducible defensins was detected, suggesting a deficient mucosal barrier function [7]. In addition, patients with Crohn's disease carrying the NOD2 mutation show an additional defect in human defensin (HD) 5 and 6 induction in inflamed ileal tissue [21,22]. In summary, these studies argue for a role for antimicrobial peptides in the course of IBD. In a recent publication Hase *et al.* [23] report cathelicidin peptide expression in colonic tissue from four patients with ulcerative colitis. However, the expression of cathelicidin in patients with ileal or colonic Crohn's disease has not been investigated. The potent antimicrobial and inflammatory activity of LL-37, and distinct activity and regulation compared to defensins, makes it important to understand expression of cathelicidin in IBD. This study focuses on the expression of cathelicidin in colon and ileal epithelium of patients with ulcerative colitis or Crohn's disease.

Materials and methods

Patients

Biopsy material was obtained during colonoscopy from a total of 89 patients after informed consent and study approval by the local ethics committees (Stuttgart, Würzburg). Twenty-eight control patients (mean age, 36.6 years) were investigated for anaemia, polyps, tumour or post-surgical follow-up. Thirty-four patients (mean age, 41.6 years) with colonic manifestation of Crohn's disease and 27 patients (mean age, 40.2 years) with ulcerative colitis underwent colonoscopy due to acute flares or cancer surveillance. Diagnosis was based on standard criteria using radiological and endoscopic findings in every case [7]. Three to four biopsies were taken from random areas of the colon in controls. In the case of IBD, two biopsies were taken from both macroscopically inflamed and non-inflamed colon segments. In addition, ileal biopsies were obtained from non-inflamed mucosa from nine control patients and from non-inflamed ($n = 7$) and inflamed ($n = 10$) ileal mucosa from Crohn's disease patients. Samples were immediately snap frozen in liquid nitrogen. Study patients were treated according to their clinical status. Remission maintenance included the administration of aminosalicylates and azathioprine, whereas acute flares necessitated steroid pulse therapy in Crohn's disease and ulcerative colitis.

RNA preparation and reverse transcription

Frozen biopsies from 64 patients (21 Crohn's disease, 15 ulcerative colitis, 28 controls) were disrupted in 1 ml Trizol (Invitrogen; Carlsbad, California, USA) until complete fragmentation as described previously [7]. Total RNA was extracted according to the supplier's protocol. RNA quality was determined by electrophoresis and quantified by photometry. Subsequently, 2 µg RNA was reverse transcribed with oligodT primers and 200 U Superscript (Gibco BRL, Karlsruhe, Germany).

Real-time RT-PCR assays

For quantification the expression of cathelicidin and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured in triplicates by real-time RT-PCR using a BioRad iCycler (BioRad, Hercules, California, USA). Primers and fluorogenic probes for cathelicidin and GAPDH have been described previously [8]. Cathelicidin and GAPDH expression was measured by Ct and corresponding transcript numbers were read off standard curves as reported previously [8]. In addition, CD4 expression levels were quantified in 12 Crohn's disease patients in inflamed and non-inflamed colon mucosa using a Taqman real-time PCR assay (Applied Biosystems, Foster City, California, USA).

Since in real-time PCR there is no established housekeeping gene for quantification of gene expression in biopsy material from inflamed and non-inflamed mucosa, relative levels of cathelicidin and CD4 transcription were calculated using two methods: gene transcript numbers were normalized to the amount of total RNA as recommended previously [7,21,24]; and, in addition, gene expression levels were normalized to GAPDH expression.

Mutation analysis

In patients with Crohn's disease genotyping of genomic and/or cDNA for the functionally relevant NOD2 mutations (SNP8, SNP12, SNP13) was performed using TaqMan technology (Applied Biosystems) as described previously [21]. In brief, amplification reactions (25 µl) were carried out with 20 ng of template DNA, 1 × Universal Master Mix buffer (ABI), 900 nM of each primer and 200 nM of each fluorogenic probe. Detection of fluorescence signals was performed using the ABI PRISM 7700 detection system and the results were analysed as described before by use of the Sequence Detection System (SDS) Software Version 1.7 (ABI) [21].

Immunohistochemistry

For detection of cathelicidin LL-37 peptide expression colon biopsy material was obtained from 25 patients with

known inflammatory bowel disease (13 ulcerative colitis, 12 Crohn's disease). Serial sections were evaluated for inflammatory activity and then stained with a specific LL-37 polyclonal rabbit antiserum as described earlier [8,25]. As a positive control, sections from healthy, uninflamed colorectal mucosa were used. As a negative control the primary antibody was pre-absorbed with excess amounts of the synthetic LL-37 peptide as described [8].

Cell culture and stimulation experiments

For in-vitro stimulation experiments HT-29 colon cells (ATCC HTB-38) were cultured in RPMI medium (Life Technologies) supplemented with 10% FCS, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg streptomycin at 37°C in the presence of 5% CO_2. Cells were grown to 60–70% confluence and subsequently stimulated with tumour necrosis factor-α (TNF-α) (20 ng/ml; Chemicon, Temecula, California, USA), interferon-γ (IFN-γ) (10 ng/ml; Roche, Indianapolis, Indiana, USA), LPS (500 ng/ml; Sigma, St. Louis, Missouri, USA), interleukin-12 (IL-12) (20 ng/ml), IL-4, IL13 (50 ng/ml) (all from R&D, Minneapolis, Minnesota, USA) and butyrate (2 mM, Sigma) for 24 h. In addition, HT-29 cells were incubated with butyrate after a 24 h pretreatment period with TNF-α, IL-4 or IL-13. Furthermore, colon cells were incubated with butyrate in combination with different cytokines. Cells were harvested and RNA isolated using Trizol (Invitrogen). All RNA material was denatured and submitted to real-time RT-PCR using an ABI PRISM 7700 detection system. Expression of cathelicidin and the housekeeping gene GAPDH were measured in triplicate from two to four independent experiments. Fold induction relative to the vehicle treated control was calculated using the $2^{(-\Delta\Delta Ct)}$ method, where $\Delta\Delta Ct$ is $\Delta Ct_{(stimulant)}-\Delta Ct_{(vehicle)}$, ΔCt is $Ct_{(cathelicidin)}-Ct_{(GAPDH)}$ and Ct is the cycle at which the detection threshold is crossed.

Statistical analyses

All statistical analyses were performed using SigmaStat 2.03 (SPSS Inc., San Rafael, California, USA). Student's paired *t*-test was used for analyses of data obtained from experimental studies, the Mann–Whitney rank sum test and the Kruskall–Wallis ANOVA were used for statistical comparison of grouped patient data.

Results

Cathelicidin expression is increased in ulcerative colitis but not in Crohn's disease

In biopsy material from 23 of the 28 control patients and from all except two samples from IBD patients cathelicidin mRNA was detected by real-time RT-PCR. Overall, highly variable expression levels were observed when comparing individual patients. Intra-individually, however, comparable cathelicidin transcript levels were detected in biopsies taken.

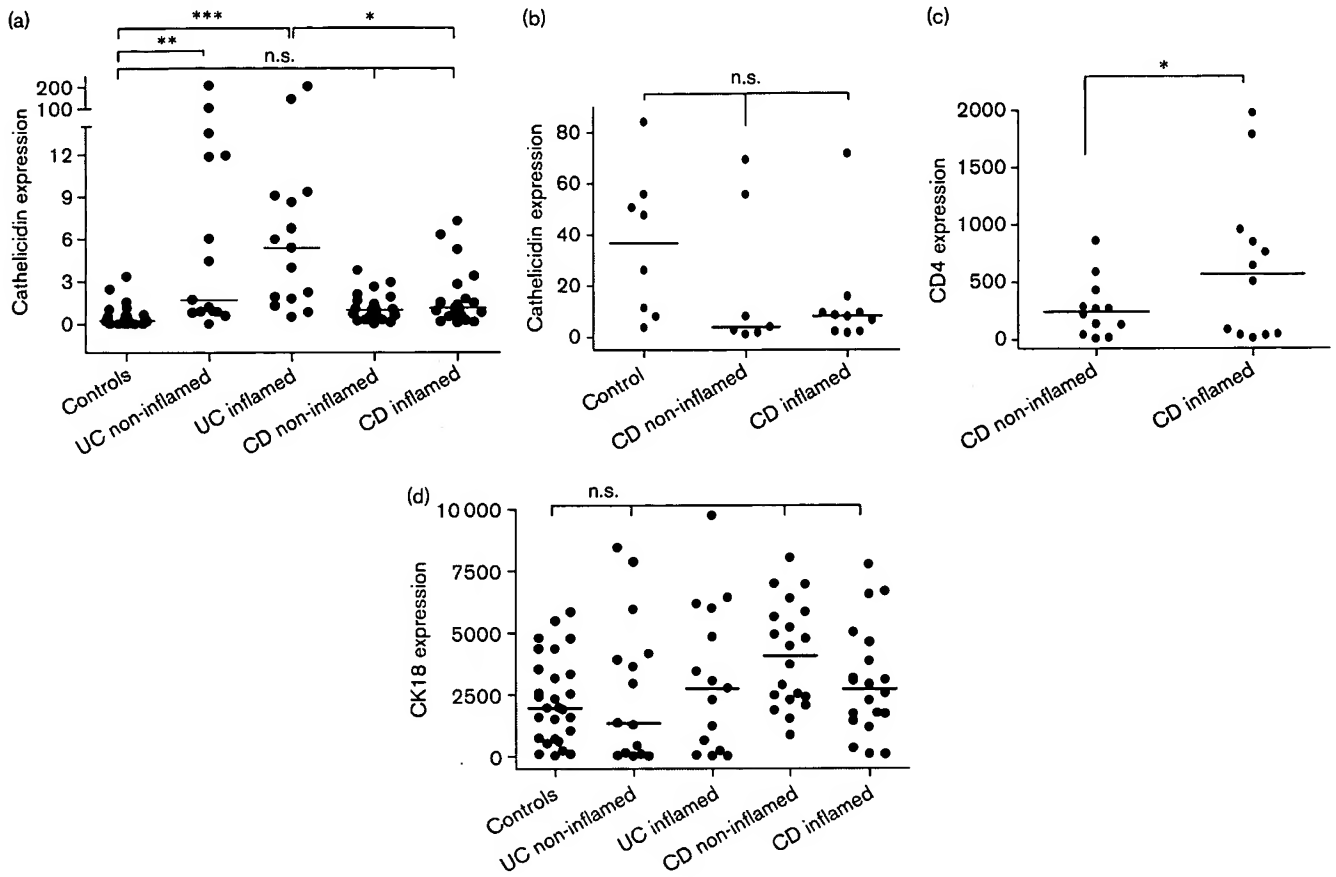
In ulcerative colitis, significantly higher cathelicidin expression levels were observed when comparing samples from non-inflamed mucosa with non-inflamed tissue from control patients ($P = 0.003$; Fig. 1(a)). This difference was even more pronounced when inflamed mucosa from ulcerative colitis patients was compared with healthy control biopsy material ($P < 0.001$). In addition, cathelicidin expression levels in inflamed and non-inflamed colon mucosa of ulcerative colitis patients were significantly higher than in inflamed or non-inflamed Crohn's disease patients respectively ($P < 0.05$). No difference was detected when non-inflamed and inflamed mucosa from ulcerative colitis patients was compared. The results from two ulcerative colitis patients are displayed as additional data points because cathelicidin expression in their non-inflamed and inflamed tissue sample was considerably higher (relative cathelicidin expression: non-inflamed mucosa 204 and 102; inflamed 201 and 141, respectively) than the other patients. These two patients were excluded from subsequent statistical analyses.

In contrast, cathelicidin expression levels did not differ when inflamed or non-inflamed mucosa from Crohn's disease patients was compared with non-inflamed healthy mucosa from controls (Fig. 1(a)). The difference of cathelicidin expression observed in biopsy material from inflamed colon mucosa versus non-inflamed mucosa in Crohn's disease was marginal and failed to reach statistical significance ($P = 0.072$). Mutation analyses identified five Crohn's disease patients with NOD2 mutations (four SNP 13, two SNP 8, one SNP 12). However, no significant change in cathelicidin transcript levels were detected in these patients compared to control mucosa (data not shown). We further analysed mucosal cathelicidin expression in the terminal ileum of Crohn's diesease patients. However, as in colon mucosa, no statistical difference in cathelicidin expression levels was observed comparing non-inflamed mucosa from control patients with either inflamed or non-inflamed ileal mucosa from Crohn's disease patients (Fig. 1(b)).

To evaluate the degree of inflammation in macroscopically non-inflamed and inflamed colon biopsies from Crohn's disease patients CD4 expression levels (as a surrogate marker for infiltrating immune cells) were evaluated [26]. Indeed, inflamed mucosa showed significantly higher expression of CD4 compatible with an increased inflammatory response (Fig. 1(c)). As mentioned above, however, this did not translate in enhanced cathelicidin expression in inflamed colon mucosa in Crohn's disease, which strongly suggests a dissociation of cathelicidin expression and inflammatory infiltration.

As described above, cathelicidin expression levels were normalized to the amount of total RNA in the investigated samples as suggested by Bustin [24]. When cathelicidin expression was normalized to the level of

Fig. 1



(a) Cathelicidin expression in colon mucosa from patients with ulcerative colitis (UC), Crohn's disease (CD) and control patients. Biopsy material from macroscopically inflamed and non-inflamed UC and Crohn's disease mucosa was evaluated by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). In addition, macroscopically healthy mucosa from non-IBD patients was analysed. (b) Cathelicidin expression in ileal mucosa from patients with Crohn's disease. Biopsy material from macroscopically inflamed and non-inflamed ileal mucosa was evaluated and compared to healthy controls. (c) CD4 expression as a surrogate marker for inflammatory cell infiltration in Crohn's disease patients. Colon mucosal biopsies were taken during endoscopy and CD4 expression was determined in inflamed and non-inflamed mucosa. (d) CK18 expression in investigated colon biopsy material. Colon biopsies from the different patient groups were analysed for the expression of CK18, a surrogate marker for epithelial cells, by real-time PCR (bars represent median expression; $*P<0.05$; $**P<0.01$; $***P<0.001$; n.s. not significant).

GAPDH in the respective sample similar results were observed: non-inflamed and inflamed colon mucosa in ulcerative colitis showed increased expression of cathelicidin ($P < 0.001$; data not shown). Again, no difference in cathelicidin expression was detected when comparing non-inflamed and inflamed mucosa from Crohn's disease patients when expression was normalized to GAPDH (data not shown).

Cathelicidin LL-37 peptide is predominantly expressed in colon epithelial cells [8,23]. To further exclude the possibility that biopsy samples differed in the amount of adjacent epithelial cells we performed real-time RT-PCR to investigate the expression of cytokeratin 18 (CK18), an epithelial marker for colon epithelial cells [27]. However, CK18 expression did not differ significantly between the patient groups analysed (Fig. 1(d)).

Cathelicidin LL-37 peptide expression in colon mucosa in IBD

As described previously, a typical staining pattern for cathelicidin expression was observed in healthy colon mucosa: differentiated colon epithelial cells at the top of the colonic crypts strongly express cathelicidin whereas epithelial cells in the deeper crypt showed no staining. In biopsies obtained from patients with IBD with differing inflammatory activity no significant alteration in this staining pattern was observed. Epithelial cells at the top of the colonic crypts expressed cathelicidin in a granular pattern (Fig. 2). However, expression was accentuated at the base of the colonocytes whereas in healthy epithelium cathelicidin staining was detected with a diffuse pattern without specific intracellular localization (Fig. 2). In addition, no significant difference in the epithelial staining was observed when inflamed and

non-inflamed mucosa from patients with ulcerative colitis or Crohn's disease was compared.

Cytokines do not affect cathelicidin expression in colon epithelial cells

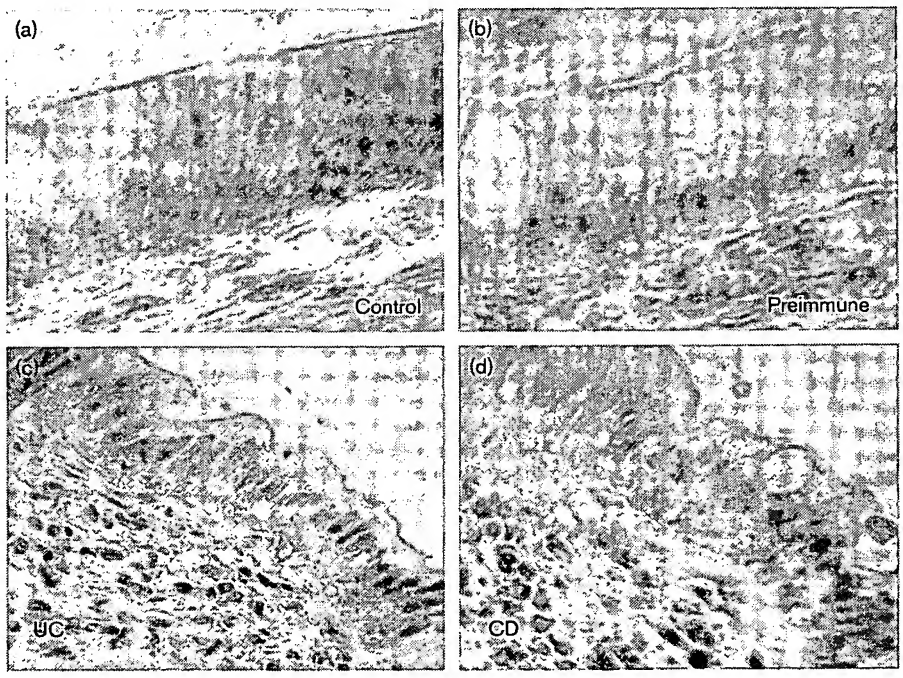
To elucidate the mechanism behind the increased cathelicidin mRNA expression in colon mucosa of ulcerative colitis patients cell stimulation experiments were performed. HT-29 colon epithelial cells were stimulated with different cytokines alone or in combination with the short-chain fatty acid butyrate, a known inducer of cathelicidin expression in these cells [8]. However, TNF-α, IFN-γ, LPS, IL-12, IL-4 and IL-13 in concentrations previously shown to exert effects on colonocytes had no effect on cathelicidin expression in HT-29 cells (data not shown, and Schauber *et al.* [8]). In addition, pre-incubation or simultaneous stimulation with IL-4, IL-13 and TNF-α did not affect butyrate-induced cathelicidin induction in these cells (Fig. 3).

Discussion

Cathelicidins are important effector molecules in innate immunity with direct antimicrobial effects on various pathogens [11]. There is evidence that cathelicidin plays an important role in the pathophysiology of human disease such as Kostmann's syndrome (a severe congenital neutropenia with chronic gingivitis due to the lack of cathelicidin in immature neutrophils) and atopic dermatitis [28,29]. Furthermore, Iimura *et al.* [30] recently showed that colon epithelial cathelicidin expression is critical for barrier function, bacterial adhesion and surface epithelial cell damage *in vivo*. Here we report the first study on the expression of human cathelicidin in colon and ileal mucosa of patients suffering from ulcerative colitis or Crohn's disease.

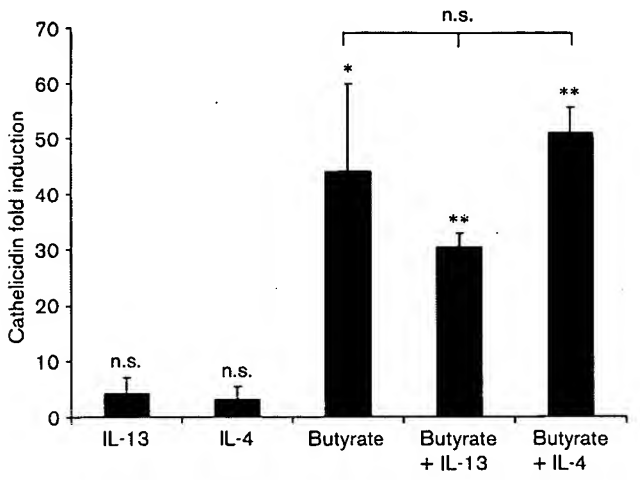
In accordance with previous reports on epithelial antimicrobial peptide expression in IBD differential expression of human cathelicidin was observed. While inflamed and non-inflamed colon mucosa in ulcerative colitis patients showed increased expression of cathelicidin mRNA, in Crohn's disease no induction compared to non-inflamed healthy colon mucosa was seen. Increased HBD-2 and HBD-3 expression in inflamed mucosa in ulcerative colitis patients was reported recently [7]. In contrast, the expression of these defensins was not induced in inflamed lesions in colonic Crohn's disease which might contribute to a defective antimicrobial barrier. Similar to increased HBD-2 and HBD-3 expression reported in non-inflamed ulcerative colitis mucosa [7], elevated cathelicidin expression was observed in the

Fig. 2



Expression of cathelicidin in epithelial cells in colonic biopsies from IBD patients. Cathelicidin expression was detected in colorectal biopsy specimens from healthy individuals by staining serial sections with the polyclonal antibody for LL-37 (a). Sections are shown at magnifications of × 250. As a negative control the primary antibody was pre-absorbed with the synthetic LL-37 peptide (b). In patients with ulcerative colitis cathelicidin was detected in epithelial cells with a granular staining pattern at the base of the colonocytes (c). A similar staining pattern was observed in patients with Crohn's disease (d).

Fig. 3



Induction of cathelicidin expression *in vitro*. HT-29 colon epithelial cells were stimulated with various cytokines alone or in combination with the short-chain fatty acid butyrate. After 24 h, total RNA was isolated and cathelicidin expression evaluated by real-time PCR. Fold induction relative to the vehicle stimulated control was calculated using the $2^{-\Delta\Delta Ct}$ method. Results from experiments done in triplicate are displayed as mean with standard error. (*$P<0.05$; **$P<0.001$; n.s.: not significant.)

present study in macroscopically non-inflamed mucosa in ulcerative colitis.

Recently, cathelicidin expression in colonic tissue from four patients with ulcerative colitis was reported [23]. Cathelicidin expression in patients with Crohn's disease has not been studied so far. In their study, Hase *et al.* [23] studied their biopsy material by immunohistochemistry while our investigation applied quantitative analysis of transcript abundance. Sole immunohistochemical analysis of the cathelicidin peptide expression might not reflect the actual expression of cathelicidin in the intestinal mucosa since this technique is not quantitative. Furthermore, the peptide is secreted, thus raising the possibility that greater immunostaining may reflect lower secretion, not more production.

Besides epithelial cells cathelicidin peptide is expressed by various immune cells [15]. Immature bone marrow cells show strong expression of the cathelicidin gene [31]. However, expression of cathelicidin mRNA disappears during the maturation process of granulocytic cells [31]. Moreover, in peripheral blood neutrophils no cathelicidin mRNA could be detected [12,31]. In IBD tissue, $CD4^+$ T cells represent the vast majority of activated mononuclear cells infiltrating the gut [26]. Crohn's disease patients showed strong immune cell infiltration in inflamed samples as reflected by increased CD4 expression in this study. Increased CD4 expression in the absence of induced cathelicidin in Crohn's disease suggests a dissociation between regulation of cathelicidin from mucosal inflammatory response.

The expression of distinct antimicrobial peptides such as HBD-2 is regulated by inflammatory mediators such as interleukins via the Nf-κB pathway [6]. The mechanisms and signalling pathways involved in the control of cathelicidin transcription are only partly elucidated. Inflammation in colitis ulcerosa is driven by Th2 cytokines such as IL-4 and IL-13 [32]. However, in addition to previously tested inflammatory mediators [8] various factors tested in this study did not induce cathelicidin expression in cultured colon epithelial cells. Short-chain fatty acids such as butyrate induce cathelicidin expression in colon epithelial cells [8,23]. As the colonic mucosa in ulcerative colitis is simultaneously exposed to butyrate and inflammatory stimuli we analysed the effect of combinations of different stimuli on cathelicidin expression in colon cells. However, IL-4 and IL-13 did not enhance cathelicidin induction by the short-chain fatty acid butyrate. Interestingly, in atopic dermatitis – another chronic Th2-driven inflammatory disease – IL-4 and IL-13 block the induction of human β-defensin 2 (HBD-2) expression in keratinocytes [33]. This leads to decreased HBD-2 peptide expression and is accompanied by increased susceptibility to bacterial infection [28]. This discrepancy – decreased antimicrobial peptide expression in Th2-driven skin disease and elevated cathelicidin expression in Th2-driven colon disease – suggests diverse regulatory mechanisms in different epithelial tissues.

It remains to be shown that a relative increase in cathelicidin expression in ulcerative colitis or a defective induction of cathelicidin expression in Crohn's disease actually translates into enhanced or diminished antimicrobial activity. Cathelicidin is stored in its pro-form in colon epithelial cells and antimicrobial activity is dependent on processing and secretion of the active peptide [25,34]. Elevated cathelicidin mRNA expression suggests increased cathelicidin production while the mucosal cathelicidin staining pattern suggests enhanced secretion or a translational defect in ulcerative colitis. Furthermore, on the skin surface LL-37 peptide is processed after secretion into multiple novel antimicrobial peptides [35]. The products of post-secretory processing of LL-37 in the colon lumen in healthy individuals and in IBD patients are unknown.

In summary, we report elevated cathelicidin expression levels in colon mucosa from patients suffering from ulcerative colitis. Cathelicidin expression is induced in inflamed mucosa in particular, but a triggering factor could not be identified. In Crohn's disease cathelicidin expression in inflamed colon mucosa was not induced compared to healthy colon biopsy material. This might result in a deficient mucosal barrier function and

aid bacterial adhesion and invasion further aggravating colonic inflammation.

Acknowledgements

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Conflict of interest

None declared.

Authors' contributions

J. Schauber (JS), E. Stange (ES), W. Scheppach (WS), R. Gallo (RG) designed the study. JS, D. Rieger (DR), F. Weiler (FW), J. Wehkamp (JW), M. Eck (ME) carried out patient sample analysis. K. Fellerman, ES, WS, JS, JW did the patient sampling. JS, DR, FW, JW did the data analysis. JS, ES, RG prepared the manuscript.

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Skin specific defects in cathelicidin antimicrobial peptide expression of subjects with atopic dermatitis and psoriasis

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Antimicrobial peptide expression is low in lesional skin of patients with atopic dermatitis (AD) but elevated in lesions of patients with psoriasis (PS).As part of the NIAID funded Atopic Dermatitis Vaccinia Network, the objective of our study was to determine if this local dysregulation was also reflected in systemic expression of cathelicidin. Saliva, blood and 2mm punch biopsies of uninvolved skin were collected from 14 subjects with PS, 9 AD, and 14 normal controls (NL). AD and PS subjects also received punch biopsies of lesional skin. Cathelicidin mRNA was measured by Q-RT-PCR and was highest in lesional PS with a median value of 2.63 relative copy numbers (RCP), significantly higher than lesional AD skin (.352 RCP) and NL skin (.064 RCP), (p=.02). Non-lesional skin of PS also trended higher than non-lesional AD skin and NL with median values of .274 RCP,and .051 RCP respectively (p=.06). Saliva cathelicidin was measured by ELISA to evaluate expression by a different epithelial cell type and was not significantly different in PS or AD compared to NL (37.1units vs.13.3 units vs. 37.6 units). Similarly, blood neutrophil cathelicidin analyzed by FACS did not show any statistical difference in mean fluorescent intensity between the PS, AD and NL. Pearson correlation analysis revealed no correlation between cathelicidin levels in the skin with blood or saliva. These results confirm previous studies showing cathelicidin is induced in PS lesional skin and that AD is not increased as expected with inflammation. New findings reveal that cathelicidin in nonlesional PS skin is increased, suggesting generalized upregulation in skin. The lack of correlation between skin, saliva and blood of all groups suggests that there is a skin specific dysregulation of cathelicidin in AD and PS patients that does not affect their saliva or blood. These observations support the clinical findings of susceptibility to infection only in the skin of AD, and support the feasibility of skin-specific modulation of antimicrobial peptide expression.

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Rosacea: Is it necessary to have photoprotection?

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Etiology of rosacea remains unknown. Pathogenesis includes increased blood flow to the dermis, and pilosebaceous unit abnormalities.In some patients it is clear the relationship between rosacea and exposure to heat.Although apparently sun exposure plays a role, this concept has been challenged since no relationship has been found between skin sun damage and rosacea in recent observations. Nevertheless general textbooks of dermatology recommend photoprotection which at this moment, is exerted almost always with topical sunscreens. We feel that applying a layer of any topical cream vehicle can increase the skin temperature and that this fact may lead to worsening of the disease or at least to difficulties in management. In order to prove that, 8 patients with rosacea had their skin temperature measured with a infrared camera (FlexCam-S Infrared Solutions Plymouth MN) before and five minutes after the application of a cream containing a sunscreen.The thermal image showed that over the central part of the face there was a significant increase in temperature between the pre and post readings (p= 0.01); this was not true for reading in lateral aspects of face or in normal people. Given our findings in this study regarding temperature augmentation after cream application, we suggest that the topical application of sunscreens and as a matter of fact of any other cream should be avoided in patients with rosacea. Of course if a patient strongly needs sun protection for other reasons regardless of the presence of rosacea it should accepted that this could be detrimental for the control of rosacea.In the long run using a sunscreen could be very expensive especially for people in third world countries but when the relation cost/effectiveness is high their use is justified. We would like to stress the point that this fact is questionable in rosacea.

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Alemtuzumab in patients with erythrodermic cutaneous T-cell lymphoma (E-CTCL)

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The anti-CD52 monoclonal antibody alemtuzumab has been shown to be effective in the treatment of hematological malignancies. We conduct a phase II study to evaluate alemtuzumab in pts with advanced stage CTCL. Preliminary results of this ongoing phase II trial and the clinical use of alemtuzumab showed efficacy particularly in pts with E-CTCL. Alemtuzumab was IV administered at a dose of 30 mg t.i.w. for 4 weeks followed by SQ administration for 8 weeks with unchanging dosage and schedule.19 pts with refractory or relapsed E-CTCL have been treated to date. The ORR was 79% (15 pts) with CR in 47% (9) and PR in 32 % (6) of patients clearing effectively circulating Sézary cells. 4 pts (21%) developed PD with the development of cutaneous tumors in one pt despite complete clearing of circulating Sézary cells. Median response duration was 7 months (range, 1-39 months). The median overall survival of all pts was 18 months (range, 1-50 months). 10 pts (53%) have died, 7 (37%) attributable to MF. Minimal residual disease was detected in 11 patients who achieved CR and PR. Treatment was well tolerated, with the majority of toxicities being Grade 2 in severity and transient. The major side effect was T-cell depletion (lymphopenia Grade 4) with a risk of infectious complications in 4 pts (Grade 2 and 3) and neutropenic fever in one patient. 5 pts developed grade 3 or 4 leukopenia with pancytopenia in 2 pts requiring withholding and/or discontinuation from treatment. One pt developed a second B-cell lymphoma 6 months after completing alemtuzumab. One pt died of aplastic anemia. Common cytopenias and prolonged immunosuppression required prophylactic treatment with TMP-sulfa, acyclovir, and diflucan until immune reconstitution. Alemtuzumab shows promising clinical activity in pts with E-CTCL with acceptable toxicities. Combined strategies with alemtuzumab may achieve molecular remissions with longer response durations in E-CTCL.

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Hydrochlorothiazide as a putative risk factor for cutaneous T-cell lymphoma

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Mycosis fungoides (MF), the most common cutaneous T-cell lymphoma (CTCL), is hypothesized to result from chronic antigen stimulation and has an associated class II HLA-DR5,DQB*03 predisposition. To study risk factors associated with MF and other CTCLs, a Microsoft access database was established from records of 1180 CTCL patients evaluated after 1987 to the present. In a retrospective analysis, 200 CTCL patients who reported a history of hypertension were predominantly MF stage IA (43%) or IB (28%), IIA(6%), IIB(5%), III (6%), IVA,B (4%) or other CTCL (8%). Of the 200, 87 or 44% took hydrochlorothiazide (HCTZ) and 77% of 87 started HCTZ prior to developing CTCL. The proportion of patients taking HCTZ was higher than that of other anti-hypertensives, including beta-blockers (15.5%), calcium channel blockers (14.5%), angiotensine-converting enzyme inhibitors (12.5%), angiotensin II receptor blockers (7.5%), other diuretics (5.5%), or unknown (1%) (p<0.0001,chi-square goodness of fit test). Median duration of HCTZ treatment prior to a biopsy diagnosis of MF/CTCL was 5 years (range 0-28 yrs). Twenty-one (24.1%) patients started HCTZ within 1 year of developing their first skin rash, and five with preceding MF or 5.7% worsened when HCTZ was started. Sixteen (18.4%) patients had complete resolution or marked improvement after stopping HCTZ therapy. One MF patient with erythroderma fulfilled Koch's postulate. She started HCTZ 4 years before diagnosis, cleared after stopping HCTZ, restarted it 4 yrs later and relapsed within 4 months. While furosemide has been associated with sub-acute lupus and Ro/La antibodies, the more common diuretic, hydrochlorothiazide, appears to have a role in triggering MF.

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Sebum excretion in response to chronic stress: beyond the myth and into reality

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It is generally accepted not only by sufferers but Dermatologists that stress plays a determinant roll on skin disease affecting the amount of naturally occurring substances like sebum, the aim of this study is to dilucidate and to valuate in an objective manner the assumed association between chronic stress and facial sebum, exploring innovating techniques for skin biometry in an observational, analytic, prospective study. We made clinical evaluations and apply stress questionnaires on a volunteer sample of 56 individuals exposed to high levels of stress, either healthy or with mild degree acne (comedogenic or mild papulo-pustular), and measure facial sebum. High level of stress was found in 60.7% (34 individuals), there was no correlation between mean facial sebum and chronic stress (R= 0.157, P= 0.4249), mean surface sebum was similar between healthy individuals and individuals with mild acne, we found a higher level of sebum excretion in male compared to female participants (4.34 vs 14.36%, respectively; P= 0.023). In a prospective evaluation after an acute effective stressor stimulus we still found no evidence of a correlation between mean facial sebum and chronic stress. Our study suggests that chronic stress does not produce a clinical or statistical change in sebum on skin surface and, more important, it is not a determinant factor for skin disease, even with the presence of acute stress peaks.

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The cutaneous renin angiotensin system: a proinflammatory pathway in pityriasis rubra pilaris?

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Pityriasis rubra pilaris (PRP) is a molecularly undefined but clinically distinct, chronic inflammatory skin disorder of unknown etiology. Whereas a number of detailed observations and intriguing hypotheses on the molecular underpinning of PRP have been made, a comprehensive list of genes and pathways that correlate with the unique clinical picture of PRP is still elusive. We have taken the approach of gene expression microarray analysis of skin biopsies from patients with PRP. Adult patients with Type I disease (Griffith classification) who were found by history and physical exam to likely suffer from PRP were asked to participate in an IRB approved research study. Biopsies were collected from involved and adjacent uninvolved skin. Differentially expressed genes were subjected to gene function analysis using the GO Ontology Database and pathway analysis using the Ingenuity Pathways software. The most significantly enriched GO categories reflected the hyperproliferative nature of PRP and included genes for keratin 16, involucrin, transglutaminase 1, cornifin and others. We also found STAT1 to be upregulated, a transcription factor recently identified to be a marker of hyperproliferative skin disease. In addition, preliminary pathway analysis suggests a role for the cutaneous renin angiotensin system (RAS) in the pathogenesis of PRP and we hypothesize that RAS has pro-inflammatory properties in human skin. Cross validation studies are ongoing to confirm these novel findings.

Cathelicidin deficiency predisposes to eczema herpeticum

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Background: The cathelicidin family of antimicrobial peptides is an integral component of the innate immune response that exhibits activity against bacterial, fungal, and viral pathogens. Eczema herpeticum (ADEH) develops in a subset of patients with atopic dermatitis (AD) because of disseminated infection with herpes simplex virus (HSV).

Objective: This study investigated the potential role of cathelicidins in host susceptibility to HSV infection.

Methods: Glycoprotein D was measured by means of real-time RT-PCR as a marker of HSV replication in skin biopsy specimens and human keratinocyte cultures. Cathelicidin expression was evaluated in skin biopsy specimens from patients with AD (n = 10) without a history of HSV skin infection and from patients with ADEH (n = 10).

Results: The cathelicidin peptide LL-37 (human cathelicidin) exhibited activity against HSV in an antiviral assay, with significant killing ($P < .001$) within the physiologic range. The importance of cathelicidins in antiviral skin host defense was confirmed by the observation of higher levels of HSV-2 replication in cathelicidin-deficient ($Cnlp^{-/-}$) mouse skin (2.6 ± 0.5 pg HSV/pg GAPDH, $P < .05$) compared with that seen in skin from their wild-type counterparts (0.9 ± 0.3). Skin from patients with ADEH exhibited significantly ($P < .05$) lower levels of cathelicidin protein expression than skin from patients with AD. We also found a significant inverse correlation between cathelicidin expression and serum IgE levels ($r^2 = 0.46$, $P < .05$) in patients with AD and patients with ADEH.

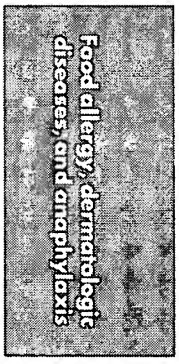
Conclusion: This study demonstrates that the cathelicidin peptide LL-37 possesses antiviral activity against HSV and demonstrates the importance of variable skin expression of cathelicidins in controlling susceptibility to ADEH. Additionally, serum IgE levels might be a surrogate marker for innate immune function and serve as a biomarker for which patients with AD are susceptible to ADEH.

Clinical implications: A deficiency of LL-37 might render patients with AD susceptible to ADEH. Therefore increasing production of skin LL-37 might prevent herpes infection in patients with AD. (J Allergy Clin Immunol 2006;117:836-41.)

Key words: Antimicrobial peptides, herpes simplex virus, atopic dermatitis, eczema herpeticum

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects approximately 17% of children and is associated with recurrent skin infections.[1,2] Recent studies have shown that the innate immune response and, more specifically, antimicrobial peptides (AMPs) are decreased in the skin of patients with AD. It has been postulated that this might explain the susceptibility of these individuals to recurrent bacterial skin infections.[3,4]

Eczema herpeticum (ADEH) is a disseminated herpes simplex virus (HSV) 1 or 2 infection that occurs in a subset of patients with AD.[5,6] Left untreated, ADEH might be fatal because of systemic viremia.[7] However, only a small subset of patients with AD have problems with recurrent viral infections, suggesting they might have an AMP phenotype distinct from that of most patients with AD. Therefore this study was conducted to investigate the role of human cathelicidin (LL-37) in controlling HSV infection and to examine differences in cathelicidin expression between patients with uncomplicated AD and patients with ADEH.



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Abbreviations used
AD: Atopic dermatitis
ADEH: Eczema herpeticum
AMP: Antimicrobial peptide
CRAMP: Mouse cathelicidin
HSV: Herpes simplex virus
LL-37: Human cathelicidin
MEM: Minimal essential medium
MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide
pfu: Plaque-forming units

METHODS

Patients

Study participants included 10 patients with AD without a history of ADEH (mean age, 29.3 ± 6.9 years) and 10 patients with AD with a history of ADEH (mean age, 39.8 ± 4.2 years). Patients were classified as having ADEH on the basis of clinical signs of ADEH, as diagnosed by a dermatologist, and a confirmation of HSV infection by means of either PCR or serology. Total serum IgE levels were measured with the UniCAP system (Pharmacia, Uppsala, Sweden). Patients in these studies were never taking oral steroids or systemic calcineurin inhibitors and stopped taking topical calcineurin inhibitors for a minimum of 1 week before enrollment. These studies were conducted according to the Declaration of Helsinki Guidelines and approved by the institutional review board at National Jewish Medical and Research Center in Denver and Ludwig Maximilian's University in Munich. All patients provided written informed consent before participation in these studies.

Skin biopsy specimens were collected from the lesional eczematoid skin rash of patients with AD and ADEH. After collection, skin biopsy specimens were fixed in formalin and archived.

Mice

BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Me). $Cnlp^{-/-}$ (Cnlp knockout) mice were obtained from R. L. Gallo (Veterans Affairs Medical Center and the University of San Diego, San Diego, Calif) and backcrossed onto the BALB/c background. All protocols with these animals were approved by the Institutional Animal Care and Use Committee at National Jewish Medical and Research Center. This institution has an animal welfare assurance number (A3026-1) on file with the Office of Protection and Research Risks.

Preparation of virus

Human herpes virus type 2 (HSV-2; a gift from Dr Adriana Weinberg, University of Colorado Health Science Center) was grown and passaged in human embryonic lung fibroblasts in Earle's minimal essential medium (MEM; GIBCO, Grand Island, NY) with 2.5% FCS (Gemini Bio Products, Woodland, Calif) and antibiotics. Freshly trypsinized lung fibroblasts were grown for 3 days to confluence and inoculated with approximately 1 plaque-forming unit (pfu) per cell in culture medium. Cells were checked daily for cytopathic effects. The culture supernatant was harvested after 48 to 72 hours of incubation at 37°C in 5% CO_2, freeze-thawed 5 times, and centrifuged for 15 minutes at 1000 rpm. For virus titration, 10-fold dilutions of stock were made, and 0.1 mL of each dilution was added to the fibroblast cell sheets in 24-well tissue-culture plates. Adsorption was allowed to take place for 1 hour at 37°C in 5% CO_2 and was followed by the addition of Earle's MEM with 2.5% FCS. Forty-eight hours after infection, medium was removed, and cells were fixed with formalin-crystal violet. Plaques were visualized on an Inverted Nikon Microscope (Nikon, Tokyo, Japan) under 1.3 × 10 magnification. Virus stocks were stored at −70°C.

Peptide preparations

Human cathelicidin (LL-37) and an irrelevant control peptide, 8044 (GLNGPDIYKGUYQFKSVEFD), were synthesized by means of solid-phase t-BOC chemistry with standard methodology and purified to homogeneity through highly purified liquid chromatography by the Molecular Resource Center at National Jewish. Peptide 8044 was chosen from a library of existing peptides for use as a control having no sequence identity with the test peptide. The identity of LL-37 was confirmed by means of mass spectroscopy. Concentrations of LL-37 and control peptides used in these experiments ranged from 0 to 100 μM.

Viral killing assay

BS-C-1 African green monkey kidney cells were seeded at 2×10^5 cells/well in 24-well plates (Becton Dickinson, Torreyana, Calif) and allowed to grow to confluence overnight at 37°C in 5% CO_2 in Earle's MEM with 10% FCS and antibiotics. To examine the effects of LL-37 and control peptide, 0 to 100 μM was incubated with 2×10^3 pfu HSV-2 for 24 hours at 37°C in a volume not to exceed 0.1 mL. Growth medium was removed from the cell sheet and rinsed once with Earle's MEM with 2.5% FCS. The virus-protein complex was added to the cells and adsorbed for 1 hour at 37°C in 5% CO_2. Growth medium was added to 0.5 mL and incubated for 24 hours for RNA analysis of HSV gene expression and 48 hours for plaque development. For the plaque assay, the medium was removed, and wells were overlaid with 0.5 mL of 4% buffered formalin and allowed to fix for 10 minutes at room temperature. The formalin was removed, and 0.5 mL of 0.1% crystal violet in PBS was added to the wells for 5 minutes at room temperature. Wells were then aspirated and air-dried for visualization of plaques. We found the most accurate results with the virus alone forming 50 to 80 plaques per well.

Keratinocyte cell culture

HaCaT cells, a human keratinocyte cell line, were cultured in Dulbecco's modified Eagle's medium (Cellgro, Herdon, Va) supplemented with 10% FCS (Gemini Bio Products) and 1% of the following until confluent: penicillin-streptomycin, l-glutamine, MEM with nonessential amino acids (GIBCO), and MEM vitamins solution (GIBCO).

HaCaT cells were infected with 0.05 pfu/cell of HSV-2 for 6 hours to evaluate the antiviral activity of LL-37. After the incubation, HSV-2 was removed, and cells were washed with medium to remove remaining HSV-2. LL-37 (0-100 μM) was added to the cells and allowed to incubate for an additional 18 hours. RNA was isolated from the cells for analysis of HSV-2 gene expression.

Murine skin explant cultures

The dorsal thorax of all mice was clipped and treated with the depilatory agent Nair to remove hair. Seventy-two hours after hair removal, mice were killed by means of CO_2 asphyxiation. Six-millimeter punch biopsy specimens were collected from the dorsal thorax and immediately placed in a 96-well plate and RPMI (Cellgro) supplemented with 10% FCS (Gemini Bio Products). Murine skin biopsy specimens were cultured in the presence of media alone or 2×10^4 pfu HSV-2 for 24 hours. After the exposure period, medium was removed, and biopsy specimens were submerged in Tri-Reagent (Molecular Research Center Inc, Cincinnati, Ohio) for RNA isolation. Three independent experiments were conducted, with a total of 15 mice in each exposure group. Data from one representative experiment are shown.

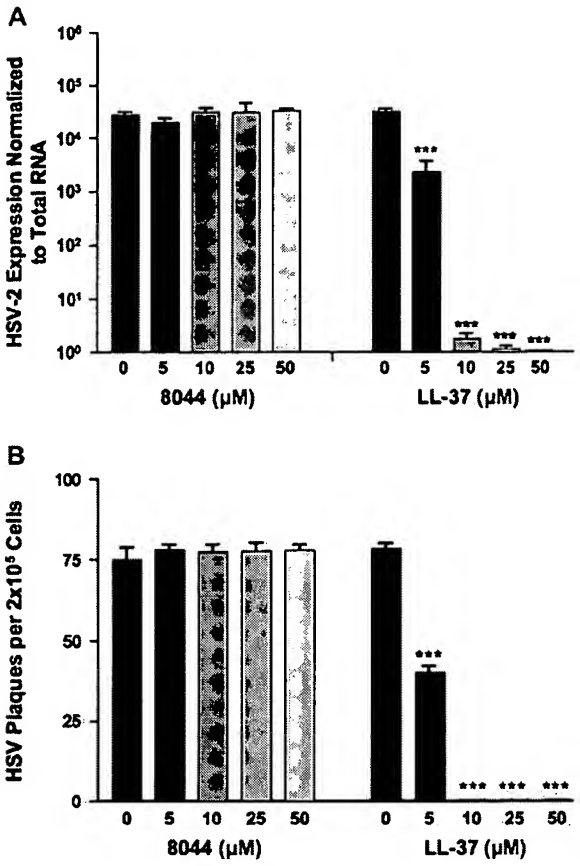
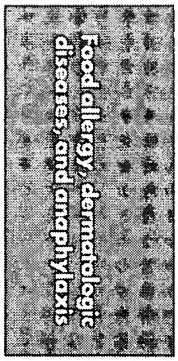


FIG 1. LL-37 exhibits antiviral activity against HSV. Physiologic concentrations of LL-37 were preincubated with HSV-2 for 24 hours and then added to BS-C-1 for an additional 24 hours to evaluate HSV-2 gene expression by means of real-time RT-PCR (A) or 48 hours to investigate functionally active virus by using a standard plaque assay (B). ***Significant difference of $P < .001$ compared with 0 μM.



Real-time RT-PCR

Total RNA was isolated from skin biopsy specimens by means of chloroform-phenol extraction and isopropanol precipitation, according to the manufacturer's guidelines (Molecular Research Center Inc). RNeasy Mini Kits (Qiagen, Valencia, Calif) were used, according to the manufacturer's protocol, to isolate RNA from cell cultures and to further purify RNA from skin biopsy specimens. Real-time RT-PCR was performed with an ABI 7000 Sequence Detection system (Applied Biosystems, Foster City, Calif), as previously described.[4] Human GAPDH and rodent GAPDH were purchased from Applied Biosystems. Primer and probe sequences for cathelicidin were designed as previously described.[3] Primer and probe sequences used to assay HSV-2 gene transcripts were as follows: forward, 5′- CGC TCT CGT AAA TGC TTC CCT -3′; reverse, 5′- TCT ACC CAC AAC AGA CCC ACG -3′. This region of the genome encodes glycoprotein D of HSV-2.[8] Relative expression levels were calculated by using the relative standard curve method, as outlined in the manufacturer's technical bulletin. Quantities of all targets in test samples were normalized to the corresponding GAPDH or total RNA levels and expressed as target gene normalized to GAPDH or target gene normalized to total RNA to allow for comparisons between samples and groups. A standard curve was generated with cDNA from purified herpes virus.

Cathelicidin protein expression

Paraffin-embedded tissues were cut into 5-μm sections, deparaffinized, rehydrated, and then stained with rabbit anti-LL-37 (5 μg/mL), as previously described.[3] All slides were coded to ensure patient anonymity, and readings were done blind so that the slide reader was unaware of the identity of the slides. Images were collected at 40× magnification, and the intensity of the immunostaining was scored on a scale from 0 to 5, with 0 indicating no staining and 5 indicating the most intense staining.

Statistical analyses

All statistical analysis was conducted with GraphPad Prism, version 3.01 (San Diego, Calif). Statistical differences in gene expression or protein staining between multiple groups was determined by using a 1-way ANOVA, and significant differences were determined by using the Tukey-Kramer test.[9] Statistical differences in total serum IgE levels were determined by using a Student t test.

RESULTS

Anti-HSV activity of LL-37

In our initial experiments we examined whether LL-37 (0-100 μM) could directly kill HSV. As shown in Fig 1, *A*, we observed a concentration-dependent inhibition of viral replication measured by means of real-time RT-PCR. Significant reduction in viral replication by LL-37 was observed with concentrations as low as 5 μM (mean, 2444 ± 1223 ng HSV/ng total RNA; $P < .001$) compared with HSV alone (mean, 32,620 ± 4061 ng HSV/ng total RNA). This was confirmed by using a standard viral plaque assay in which preincubation of HSV with 5 μM of LL-37 significantly ($P < .001$) reduced the number of plaques from 78.3 ± 1.5 (HSV alone) to 40.3 ± 1.9 (Fig 1, *B*). The control peptide 8044 possessed no antiviral activity against HSV.

Role of cathelicidins in controlling HSV replication in the skin

To examine a more physiologic condition, human keratinocyte cultures were preinfected with HSV for 6 hours and then treated with exogenous LL-37 to determine whether intracellular viral replication could be halted with physiologic concentrations of LL-37. Fig 2 demonstrates that concentrations of LL-37 as low as 25 μM (mean, 563 ± 71 ng HSV/ng GAPDH) were able to significantly ($P < .01$) reduce the levels of HSV gene expression in previously infected keratinocytes (HSV mean, 1614 ± 158).

To demonstrate the clinical relevance of LL-37 compared with other potential arms of the innate immune response in limiting HSV infection, we used mice deficient in Cnlp, the murine cathelicidin. Significantly higher levels of HSV replication were observed in skin biopsy specimens from Cnlp knockout mice (BALB/c background; 2.6 ± 0.5 pg HSV/pg GAPDH, $P < .05$) compared with that seen in skin biopsy specimens from wild-type BALB/c mice (0.9 ± 0.3 pg HSV/pg GAPDH, Fig 3), suggesting that cathelicidins play an

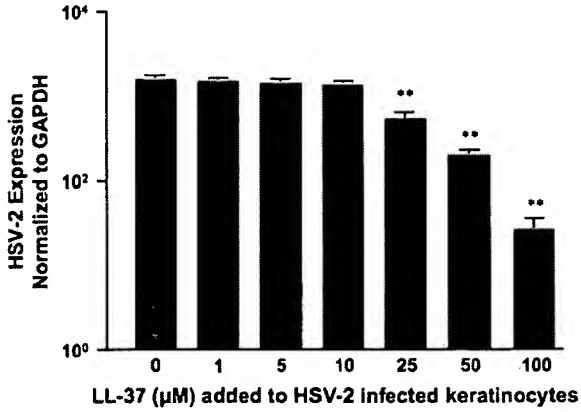


FIG 2. Exogenous LL-37 rescues HSV-infected keratinocytes. Human keratinocytes were infected with 0.05 pfu/cell HSV-2 for 6 hours and then treated with physiologic concentrations of LL-37 for an additional 18 hours. RNA was isolated from the cells, and the levels of HSV-2 gene expression were evaluated by means of real-time RT-PCR. **Significant difference of $P < .01$ compared with HSV alone.

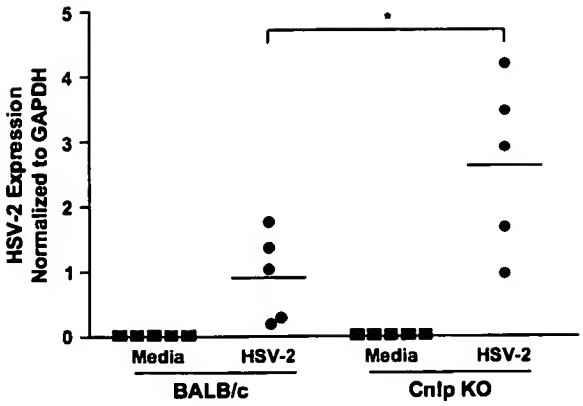


FIG 3. Essential role of cathelicidins in controlling HSV replication in the skin. Skin biopsy specimens from BALB/c (n = 5) and Cnlp knockout (n = 5) mice were stimulated with HSV-2 for 24 hours and evaluated for HSV-2 gene expression. RNA was collected from the tissue, and the levels of HSV-2 were evaluated by means of real-time RT-PCR. *Significant difference of $P < .05$.

important role in controlling HSV skin infection. These data are representative of 3 independent experiments with a total of 15 mice.

Deficiency of LL-37 in the skin of patients with ADEH

Skin biopsy specimens were collected from the skin lesions of adult patients with AD and patients with ADEH. Biopsy specimens were stained with a polyclonal antibody specific for LL-37 to investigate cathelicidin expression. All slides were coded before analysis, and readings were done blind so that the slide reader was unaware of the identity of the slides. Fig 4, *A*, shows that skin sections from patients with AD exhibited more staining for cathelicidin than skin lesions from patients with ADEH. The composite data for cathelicidin immunostaining in all samples is shown in Fig 4, *B*. The intensity of cathelicidin staining in ADEH skin lesions was significantly ($P < 0.05$) lower than that seen in skin lesions from patients with AD.

Correlation between serum IgE level and cathelicidin expression

Previous studies have suggested that T_H2 cytokines could inhibit cathelicidin production.[10] Because of the limited amount of archived tissue available, we were unable to investigate potential differences in IL-4 and IL-13 expression between patients with AD and patients with ADEH. However, IL-4 and IL-13 are T_H2 cytokines essential in the production of IgE.[11] Therefore we examined whether increases in serum IgE levels might correlate with cathelicidin expression in patients with ADEH and patients with AD. Because antibody generation is exponential, we log transformed the serum IgE values for further statistical analysis. Using linear regression analysis, we demonstrate a significant correlation ($r^2 = 0.46$, $P < .05$) between total serum IgE levels and cathelicidin protein expression in patients with AD and patients with ADEH (Fig 5).

DISCUSSION

AMPs are an integral part of the innate immune response because they have been shown to be effective in killing bacterial[3] and viral[12] pathogens. Cathelicidin is produced by several cells in the skin, including keratinocytes, where it is induced in response to inflammatory stimuli.[13,14] On release, the cathelicidin precursor protein is processed into the biologically active AMP LL-37. In this study we demonstrate that LL-37 exhibits antiviral activity against HSV. Our previous studies have demonstrated that patients with AD, in general, have a reduced ability to generate cathelicidin in their skin compared with patients with psoriasis or allergic contact dermatitis, and this might predispose them to microbial skin infection.[3,10] However, the propensity of patients with AD to have serious skin infection, such as ADEH, has not previously been explored. Results from the current study indicate that a more exaggerated reduction in cathelicidin expression might predispose a subset of patients with AD to having ADEH. Thus there is heterogeneity in the expression of cathelicidin within AD, such that the individuals with the lowest levels are most prone to disseminated viral infection.

It was previously reported that LL-37 exhibits little activity against HSV-1 or HSV-2.[15] In contrast, we demonstrate in this study that LL-37 exhibits potent antiviral activity against HSV. In the previous study Yasin et al[15] demonstrated that 44.5 μM of LL-37 provided 28% and 46% protection against HSV-1 and HSV-2, respectively, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. In the MTT assay antiviral activity of LL-37 is determined on the basis

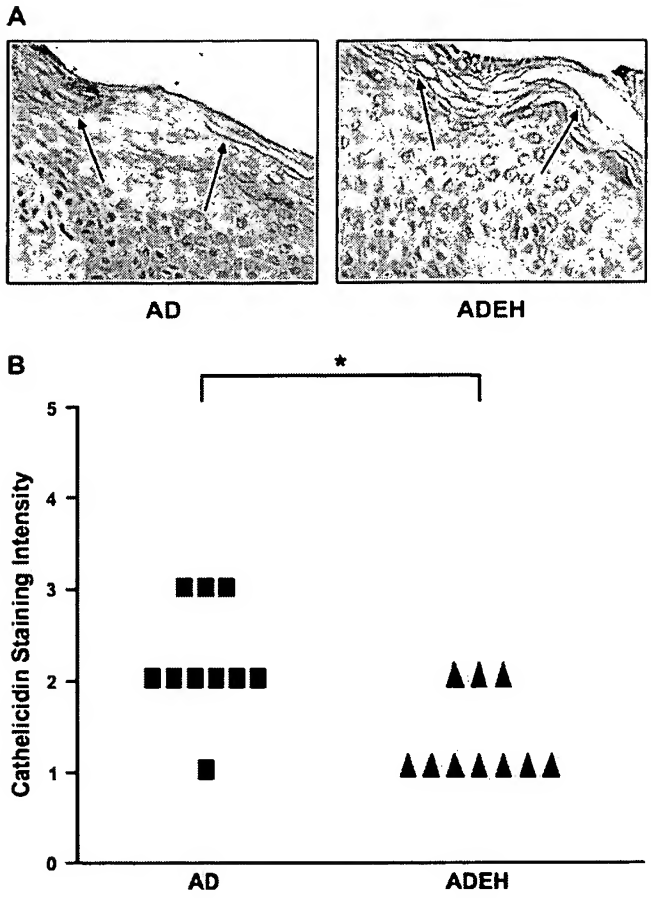


FIG 4. Expression of cathelicidin increased in AD skin compared with that seen in ADEH skin. **A**, Paraffin-embedded skin explants from patients with AD (n = 10) and patients with ADEH (n = 10) were cut into 5-μm sections and stained for human cathelicidin. **B**, The intensity of the immunostaining was visually scored on a scale from 0 to 5, with 0 indicating no staining and 5 indicating the most intense staining. *Significant difference of $P < .05$.

of increases in cellular proliferation compared with cells treated with virus alone. In our current study we measured HSV replication by evaluating glycoprotein D gene expression using real-time RT-PCR. Directly measuring the virus activity rather than cellular proliferation provides a more direct and appropriate measurement of the antiviral activity of LL-37 against HSV, accounting for differences between our results and those of Yasin et al.[15] Our observation is further supported by Gordon et al,[16] who recently demonstrated that LL-37 exhibits antiviral activity against HSV-1 in corneal and conjunctival epithelia.

In this study we demonstrate that concentrations as low as 10 μM of LL-37 reduce HSV levels by more than 10,000-fold. Because psoriatic skin can contain up to 1605 μM LL-37,[3] this demonstrates that physiologic concentrations of LL-37 are effective at controlling HSV replication. This was further supported by using a more physiologic approach in which keratinocytes were preinfected with HSV for 6 hours and then incubated with LL-37 for an additional 18 hours. Again, we saw greater than a 60% reduction in the levels of HSV when as little as 25 μM of LL-37 was added to previously infected keratinocytes. The importance of cathelicidins in skin innate immune responses to HSV is also strongly supported by our current finding that skin explants from mice deficient in the cathelicidin gene Cnlp and its AMP product, mouse cathelicidin (CRAMP), sustain higher levels of HSV replication after inoculation compared with those seen in their wild-type counterparts. Mouse CRAMP is very similar to human LL-37 in structure, tissue distribution, and antimicrobial activity and is therefore a reasonable model of the human cathelicidin. The observation that CRAMP-deficient mice support a higher level of HSV replication reinforces the important effect that a cathelicidin deficiency would have on HSV skin infection in human subjects. These data support a conclusion that decreased cathelicidin expression will significantly increase the potential for disseminated skin infection to occur.

Because not all patients with AD have ADEH, we investigated the abundance of cathelicidin in the skin of patients with AD and patients with ADEH to determine whether the development of ADEH corresponded with decreased cathelicidin expression. Skin biopsy specimens were obtained from naturally induced inflammatory skin rashes of AD and ADEH. This allowed for comparisons

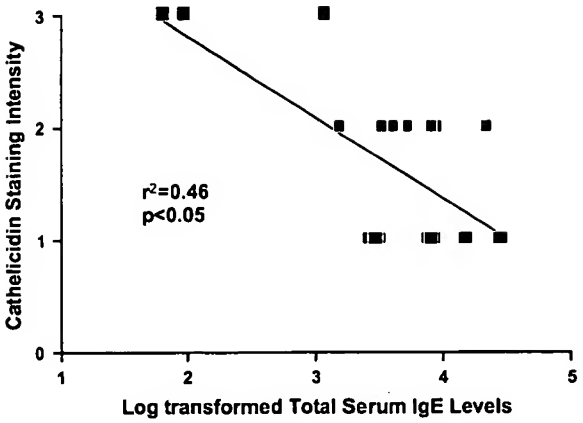


FIG 5. Correlation between serum IgE levels and cathelicidin expression in patients with AD and patients with ADEH. Serum IgE levels were determined from patients with AD (n = 9) and patients with ADEH (n = 9). Regression analysis was performed on log-transformed serum IgE values and LL-37 protein expression.

between similarly stimulated skin samples. Lesional skin from patients with ADEH exhibited significantly lower levels of cathelicidin protein than skin lesions of patients with uncomplicated AD. Our results suggest that patients with AD with the lowest levels of cathelicidin are most susceptible to development of ADEH and that lack of this molecule might serve as a biomarker for patients at risk for disseminated viral skin infection.

AD skin is characterized by the overexpression of the T_H2 cytokines IL-4 and IL-13.[17] Previously, we demonstrated that T_H2 cytokines downregulate cathelicidin.[10] However, because of the difficulty in identifying patients with ADEH and insufficient amounts of archived tissue, we were unable to investigate the levels of IL-4 and IL-13 in patients with AD and patients with ADEH for potential differences. IL-4 and IL-13 are T_H2 cytokines essential in the production of IgE.[11] Therefore the measurement of serum IgE levels might serve as a biomarker for levels of T_H2 responses. Wollenberg et al[6] and Lagace-Simard et al[18] have previously demonstrated that patients with ADEH exhibited higher total serum IgE levels than patients with AD. We confirmed this observation in the current study by demonstrating significantly higher serum IgE levels in patients with AD compared with those seen in patients with ADEH. We further determined that there is a strong correlation between the levels of serum IgE and cathelicidin protein expression in patients with AD and patients with ADEH. Therefore serum IgE levels might serve as a surrogate marker for the expression of cathelicidin in the skin of patients with AD and patients with ADEH and might separate those who are more susceptible to disseminated viral infection.

The current study therefore demonstrates that AD represents a heterogeneous population of patients expressing different levels of cathelicidin in the skin. This explains, in part, why a subgroup of patients with AD is susceptible to ADEH after HSV infection. Additionally, this study demonstrates the importance of the cathelicidin in controlling the replication of HSV in the skin. This is supported by significantly higher levels of HSV replication in the skin of Cnlp knockout mice and the significant reduction of HSV gene expression in keratinocytes treated with LL-37. Overall, these data suggest further clinical studies are warranted to examine whether augmentation of LL-37 expression in AD skin might be useful in the prevention of ADEH and the perplexing challenge of controlling microbial infection in this common skin problem.

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Expert Opinion

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Monthly Focus: Anti-infectives

Cationic antimicrobial peptides – an update

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Cationic antimicrobial peptides play a very important role in nature as a first line of defence against attack and damage. However, their application to the clinic has not been very encouraging to date. There are indications that the barriers to their success may now be eroding with companies developing peptides to be more stable, cost effective and targeted to specific indications. These include systemic infectious disease, acne, vaginitis, wound infection and inflammation. In addition, the use of such peptides as modulators of innate immunity in the treatment of infectious disease and inflammation has added a further dimension to the field.

Keywords: antibacterial, antifungal, antimicrobial, cationic peptides, innate immunity

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1. Introduction

Antimicrobial peptides are the main component of the innate immune system. They are gene encoded and present in the granules of neutrophils and secretions of the mucosal epithelia, providing the host with a repertoire of small peptides that are promptly synthesised upon induction, easily stored in large amounts and readily available for antimicrobial warfare against numerous pathogenic microorganisms, including viruses, Gram-positive and -negative bacteria, protozoa, yeast and fungi, as well as cancer cells [1-5]. These peptides have three characteristic properties: they are relatively small (20 – 46 amino acid residues), basic (lysine- or arginine-rich) and amphipathic. They may be grouped into five broad families based on structural features, namely α-helices, β-sheets, looped (connected by a single disulfide bridge), extended helices and cyclic (connected by a peptide bond) [6,7].

Clearly different from the mode of action of conventional antibiotics, which often interact with specific microbial targets, antimicrobial peptides appear to function via a selective, but not receptor-mediated, permeabilisation of microbial membranes. LL-37 is a membrane-active peptide. It increases the lamellar-to-inverted hexagonal phase-transition temperature of both phosphoethanolamine (PE) model lipid systems and *Escherichia coli* lipids, demonstrating that it induces positive curvature strain in these environments and is in favour of a toroidal mechanism [8]. This study also suggests that micelles or other small, rapidly-tumbling membrane fragments are not formed in the presence of LL-37. MSI-78, a synthetic magainin analogue developed by the Genaera Corporation, was also shown to induce positive curvature strain in lipid bilayers [9]. At lower concentrations (1 – 5%), the peptide altered the morphology of the bilayer and at higher concentrations (10 – 15%), it led to the formation of a mixture of normal hexagonal- and lamellar-phase lipids indicative of the formation of a toroidal pore [9]. Alternatively, the carpet model is used to interpret the mechanism of lytic peptides [10]. Most mammalian innate antimicrobial peptides, such as defensins and protegrins, are membrane lytic and such activity can be increased or decreased by *in vitro* alteration of the peptide sequence.

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In contrast, the activity of some antimicrobial peptides cannot be explained by membrane disruption since their abilities to depolarise bacterial membranes lack correlation with their antibacterial activities [11]. The proline/arginine-rich mammalian cathelicidin peptide PR-39 is active against Gram-negative bacterial species, enters the cell without membrane lysis and, once in the cytoplasm, binds to and inhibits the activity of specific intracellular targets essential to bacterial growth, causing cell death [12]. Studies on the interaction of indolicidin with the protozoan pathogen *Leishmania donovani* also showed extensive intracellular damage, including cytoplasmic vacuolisation and degeneration of cellular organisation, without plasma-membrane disruption as determined by transmission electron microscopy [13]. Preliminary gene profiling of *E. coli* treated with a sublethal level of cecropin A suggests that it induces a genomic response, separate from any lethal effects, on the membrane and > 26 genes (half of them with unknown function) were significantly affected simultaneously following peptide treatment [14].

Nearly two decades of extensive studies on antimicrobial peptides of innate immunity suggest that the ancient molecules have not only evolved to act against distinct microbial targets in different physiological contexts, but a few of them have also been found to modulate the immune system, providing a wealth of applications including wound healing, anti-inflammation and innate immunity stimulation to combat disease. They have become more and more attractive in offering novel templates for pharmaceutical compounds that could be effective against increasingly resistant microbes.

This review will provide an update on new developments in antimicrobial peptide research, the rationale for clinical use and the paths taken to translate these biological activities into therapeutic products by the leading peptide-development companies around the world.

2. The role of antimicrobial peptides in innate defence

In mammals the cathelicidin and defensin gene families are undoubtedly the dominant classes of the innate antimicrobial peptides [15]. Cathelicidins belong to one family of antimicrobial peptides characterised by conserved pro-peptide sequences that have been identified in several mammalian species. LL-37/human cationic antimicrobial protein-18 (hCAP-18) is the only cathelicidin found in humans. In contrast, there have been six α-defensins identified, six β-defensins and a cyclic retrocyclin (dimeric α-defensin in a cyclic structure) [7,16,17]. All classes of peptide, except retrocyclins, are expressed in inflammatory and epithelial cells. The role of these peptides in innate defence is essential and in line with their expression, both constitutive and inducible, in every niche of the human body susceptible to invasion and colonisation by pathogens. This includes skin [18] and mucosal membranes lining body cavities exposed to the exterior such as salivary glands [19], nasal mucosa [20], the respiratory [21], gastrointestinal [15] and genitourinary [22] tracts and colon mucosa [23].

Animal studies, including a recently developed human defensin-5 transgenic mouse model [24], has supported the role of peptides in innate defence. The abnormal expression of innate peptides has also been implicated in certain human diseases including eczema, atopic dermatitis, psoriasis [25,26] and morbus Kostmann, a severe congenital neutropenia [27]. Inactivation of innate peptides by multiple factors including salt concentration, overexpression of proteases and cathepsins and high levels of anionic filamentous molecules has been implicated in airway infections in the lungs of cystic fibrosis (CF) patients [28,29]. Recently, an impaired induction of human β-defensins-2 and -3 was also reported for Crohn's disease, an autoimmune disorder of excessive immunological activity with a chronic, sometimes transmural, inflammatory process within the bowel wall [30]. Such deficiency in innate peptides and other factors may have caused primary breakdown of the mucosal defence to allow an increased bacterial adherence at the intestinal mucosa leading to massive inflammation in the bowel wall [31]. Hepcidin is a group of multi-cysteine-containing antimicrobial peptides, different from defensins, identified in humans as part of the innate immune system and a key regulator of cellular iron egress [32-34]. Inappropriate production of hepcidin has been directly related to anaemia of chronic disease, a common disorder that afflicts patients with a wide variety of inflammatory conditions including arthritis, malignancies, infections and inflammatory bowel disease [35].

It has been well documented by most *in vitro* studies that both cathelicidins and defensins are multifunctional and act as signalling molecules that activate host cell processes involved in adaptive immune defence such as chemotaxis [36], anti-inflammation and antisepsis via neutralisation of endotoxin [37] and the release of cytokines and histamine [38,39]. New data has provided evidence for the role of innate peptides in wound healing. Human neutrophil defensins (HNP1 – 3) were initially shown to induce airway epithelial cell proliferation [40] and further evidence suggests that these peptides enhance airway epithelial wound closure and mucin gene expression [41]. In humans, high levels of LL-37/hCAP-18 are produced in the skin upon wounding, the highest levels being attained at 48 h postinjury, then declining to preinjury levels upon wound closure [42]. Using a non-inflammatory *ex vivo* wound-healing model, composed of organ-cultured human skin, LL-37/hCAP-18 was shown to be strongly expressed in healing skin epithelium but absent in the epithelium of chronic ulcer [42], suggesting that this peptide enhances re-epithelialisation of skin wounds and contributes to the failure of the chronic ulcer to heal [42]. The role of LL-37 is now widening to angiogenesis, a process essential for host defence, wound healing and tissue repair. Exogenously, administration of LL-37 stimulates capillary formation in a rabbit hind-limb model of ischaemia, and mice deficient in the murine analogue of LL-37/hCAP-18 demonstrated less neovascularisation of skin lesions than wild-type controls [43]. The

chemoattractant and angiogenic actions of LL-37 are thought to be receptor-mediated by interaction with formyl peptide receptor-like 1, found on macrophages, neutrophils and subsets of lymphocytes [36,43].

3. Innate immunity defence peptides derived from host proteins

It is well known that host proteins including bactericidal permeability-increasing protein (BPI) [44], lysozyme [45] and lactoferrin [46] possess active roles in innate defence. These host protein-derived active antimicrobial peptides hold great promise for the development of systemic anti-infectives and indeed, both lactoferrin- and BPI-derived peptides have already entered the drug development stage in biotech companies (see Section 4). Recently, such host proteins involved in innate defence have been extended to platelet polypeptides, chemokines and others. Human skin produces a 14.5 kDa antimicrobial ribonuclease, RNase 7, which exhibits a broad spectrum of antimicrobial activity against many pathogenic microorganisms [47]. Its expression was detected in various epithelial tissues including the skin, respiratory tract, genitourinary tract and at a low level in the gut upon bacterial challenge [47]. This is the first report demonstrating RNases as a novel class of epithelial inducible antimicrobial proteins, which may play an important role in the innate immune defence system of human epithelia.

Evidence for an active role of platelets in innate host defence was revealed recently, although the antibacterial activity of animal serum was observed nearly a century ago. Human platelets possess, and can be stimulated to release, several antimicrobial polypeptides [48]. These peptides, human platelet antimicrobial peptides (HPAPs), range 12 – 16 amino acids in length and are the truncated products of several platelet chemokines, including CXC chemokine ligand (CXCL)-4, platelet factor (PF)-4, CC chemokine ligand (CCL)-5, RANTES (regulated upon activation, normal T-cell-expressed and secreted) and full-length connective tissue activating protein (CTAP)-3, along with the CTAP-3 precursor platelet basic protein. HPAPs have potential antimicrobial activity for *E coli, Staphylococcus aureus, Cryptococcus neoformans* and *Candida albicans* [48]. Antimicrobial activity has also been described for CCL20 expressed by airway epithelial cells, which apparently shares structural and functional properties with defensins [49]. These findings suggest that some chemokines evolved the ability to contribute to innate defence in addition to their role in adaptive immunity.

Many surface epithelial cells express adrenomedullin, a peptide with many properties in common with innate antimicrobial peptides in terms of size, number of positive charges and amphipathicity [50,51]. It has good antibacterial activity against pathogens of the human skin, oral, respiratory tract and gastrointestinal tract. A significant increase in secretion of this peptide was observed upon exposure of human gastric epithelial cells to viable cells of *Helicobacter pylori, E. coli, Salmonella enterica* or *Streptococcus bovis* [50,51]. Therefore, adrenomedullin may represent a new category of antimicrobial peptides, which contributes to the mucosal host defence system.

The proteolytic degradation of haemoglobin into small biologically active peptides apparently starts inside erythrocytes, and the resulting peptides, consisting of 17 and 36 amino acids, have been demonstrated to inhibit the growth of Gram-positive and -negative bacteria and yeast in micromolar concentrations. These peptides also possess anti-endotoxin activity but are not toxic to human primary blood cells [52]. These peptides could be important effectors of the innate immune response of killing microbial invaders.

4. Commercialisation of innate immunity peptides

The current status of development programmes being pursued by specific companies (in alphabetic order) are outlined below, based primarily on website publications, public disclosures and press releases.

4.1 Adaptive therapeutics

Adaptive Therapeutics (San Diego, CA, USA) is a company built around the technology of MR Ghadiri of The Scripps Institute. This is based upon six- and eight-residue cyclic D-,L-α-peptides that act preferentially on Gram-positive and/or Gram-negative bacterial membranes compared to mammalian cells, increasing membrane permeability, collapsing transmembrane ion potentials and causing rapid cell death. The effectiveness of these antibacterial peptides has been demonstrated by efficacy against lethal methicillin-resistant *S. aureus* (MRSA) infections in mice. The advantages of cyclic D-,L-α-peptides, highlighted by the company, include proteolytic stability, ease of synthesis and a potentially vast sequence diversity. In addition, the unique abiotic structure of the cyclic peptides may contribute to a reduced emergence of drug-resistant bacteria.

4.2 Agennix

Agennix (Houston, TX, USA) has a preclinical pipeline that includes proprietary anti-infective and anti-inflammatory peptides. This includes a portfolio of 18 novel peptides developed through several generations of targeted amino-acid substitutions. The anti-infective peptides are active against a broad range of Gram-positive and -negative pathogenic bacteria including strains of MRSA and vancomycin-resistant *Enterococcus* (VRE). The peptides are also synergistic with conventional antibiotics. The lead anti-infective peptides are AGX-P24 and AGX-P35. In addition, the company's peptides have demonstrated potent anti-inflammatory activity in two separate animal models. The lead anti-inflammatory peptides, AGX-P21 and AGX-P23, are being developed for inflammatory indications. Agennix is targeting the key indication of sepsis with peptides, such as AGX-P10 and AGX-P11, that exhibit anti-infective, anti-inflammatory and

lipopolysaccharide (LPS)-binding activity. However, the primary focus of the company is towards the development of recombinant human lactoferrin (rh lactoferrin).

4.3 AM Pharma

AM Pharma (Bilthoven, the Netherlands) is developing therapeutic agents that possess both anti-infective and immunostimulating activity based upon human histatin- and lactoferrin-derived peptide sequences. This technology originated at the Free University of Amsterdam and Pharming Group NV. In addition, AM Pharma has licensed ubiquicidin-derived peptides from Leiden University. Two main areas have been identified for product development by AM Pharma; the treatment of hepatitis C, and the development of intravenous treatment and prevention of infections that may develop after orthopaedic and trauma surgery, caused by multiple resistant bacteria (such as MRSA) in particular.

4.4 Demegen, Inc.

Demegen, Inc. (Pittsburgh, PA, USA) announced in June 2003 that it has entered into a collaborative agreement with the topical therapeutic company Dow Pharmaceutical Sciences to develop certain Demegen peptides into prescription pharmaceutical products. However, Demegen is not currently developing any of its technology in-house.

The topical applications of Demegen peptides are based around the gel formulation (Demegel™) of D2A21 (a 22-residue α-helical peptide) and P113L (a 12-residue portion of histatin). D2A21 is active against many infectious agents, including multi-drug resistant strains of *P. aeruginosa* and *S. aureus* and a range of fungi (*C. albicans*, *Aspergillus niger*, *Mucor sp.* and *Trichophyton mentagrophytes*) [53,54]. Demegel™ was demonstrated to be as good as the current standards of care, as indicated by *in vitro* and *in vivo* results. The initial safety profile of the peptide suggests that it does not inhibit wound healing and is not cytotoxic to cultured keratinocyte skin cells. It is not a dermal irritant, nor does it induce contact sensitisation. The peptide is not a mammalian or bacterial mutagen. Acute and chronic systemic toxicity studies have established a safe dose, which is a multiple of the efficacious dose for Demegel. Demegen was also developing P113L in a rinse formulation for the treatment and prevention of oral candidiasis [55]. Toxicology studies and experience in the clinic with a P-113 mouth rinse for the treatment of gingivitis demonstrated the peptide to be safe and well-tolerated. This product had progressed through Phase II clinical studies and has been tested in > 300 subjects.

4.5 Entomed SA

Entomed SA (Strasbourg, France) was founded in 1999 to capitalise on the work of Jules Hoffman at the Centre National de la Recherche Scientifique (CNRS) Molecular and Cellular Biology Institute in Strasbourg. The lead compounds are based on natural peptides and small molecules derived from insects [56]. So far, Entomed has purified and identified hundreds of novel molecules that have either a broad spectrum of activity against fungi and bacteria or have antiproliferative effects. Entomed's lead peptide-based therapeutic is ETD-151 (44 amino acids containing 3 disulfide bonds), a novel analogue of heliomicin (G20A/N19R/D17N), which is a naturally occurring antifungal peptide from the haemolymph of the lepidopteran *Heliothis viriscens* [57].

The target indication for this peptide is as a systemic antifungal. The *In vitro* spectrum of activity includes all major fungal pathogens with the exception of *Candida glabrata*. ETD-151 has demonstrated efficacy in a 5-day murine *C. albicans* survival model dosing at 5 and 30 mg/kg at 6, 24 and 48 h post infection. The higher dose gave almost complete protection despite a half-life of only 5 min in the mouse. Entomed is currently seeking partners to exploit its proprietary technologies and product candidates through alliances, partnerships and licensing agreements with pharmaceutical and biotechnology companies in multiple therapeutic areas.

4.6 Genaera Corporation

Genaera (Plymouth, PA, USA) is now the owner of the peptide development programmes of Magainin Pharmaceuticals. Magainin Pharmaceuticals was founded to take forward the first commercial innate immunity antimicrobial peptide candidate based upon the initial discoveries of Michael Zasloff [58]. However, the future of pexiganan acetate (MSI-78, Locilex™), the 22-amino acid magainin peptide developed for infections of diabetic foot ulcers, seems unclear. This development candidate did not obtain US Food & Drug Administration (FDA) approval in 1999 and in conjunction with GlaxoSmithKline (GSK) a decision on its future has not been made. In 2002, Genaera entered a 3-year option agreement for its antimicrobial peptide intellectual property with DuPont (E.I. du Pont de Nemours). Genaera no longer conducts significant research and development activities in this area as a result of a reprioritisation of corporate goals.

4.7 Helix BioMedix, Inc.

Helix BioMedix, Inc. (Bothell, WA, USA) has assembled intellectual property covering a wide range of peptides of the innate immune system. Unlike other companies in the field, Helix has a diversity of structural classes including α-helical, β-sheet, linear and looped peptides in-license from Louisiana State University and the Hancock Laboratory at the University of British Columbia, and has intellectual property developed in-house. The company's library includes peptides based on crab polyphemusins, insect cecropins and melittins, cattle bactenecins, fish pleurocidins and short synthesised biologically active peptides consisting of > 100,000 distinct sequences.

The underlying properties of the peptides being exploited are antimicrobial activity and immune modulation. The company has created a diverse and extensive library of peptides in order to optimise the required attributes for each clinical application. In addition, Helix

has developed peptides specifically around sequences that are cost effective to synthesise, enabling such peptides to compete with current therapies.

HB-107 has demonstrated efficacy in rat burn wound, mouse acute wound and full thickness pig burn wound models. The peptide has completed its first gel (0.01% HB-107) formulation trial, proving it safe, efficacious and significantly better than current therapies in preclinical testing. The peptide exhibits no antimicrobial activity or cytotoxicity and has an intravenous median lethal dose (LD_{50}) > 100 mg/kg in the mouse. In a pilot test conducted by Charles River Laboratories, HB-107 appeared to speed up the regeneration of new cells (re-epithelialisation) in the process of healing burn wounds. Pigs treated with relatively low concentrations of the peptide appeared to regenerate new cells faster than those treated with a placebo gel. The 4-week study was conducted on full thickness burn wounds. The peptide was applied once-daily in gel formulation at two concentrations (0.1 and 0.5% HB-107). At both concentrations the peptide produced an improved degree of re-epithelialisation in animals after 14 and 28 days, respectively. Although only a small-scale study, regression analysis demonstrated that the peptide might be capable of reducing the time to achieve 50% re-epithelialisation in this model, from 16 to 10 days. This measurement is the standard scientific barometer for wound healing. The peptide showed no signs of toxicity at either concentration as determined by histology and extensive blood analysis. Studies on pigs are almost always the final step before wound-healing tests on humans begin. The company's business plan calls for it to license its proprietary peptides to other firms, which then conduct clinical trials and develop and market new drugs.

HB-50 is a broad-spectrum antimicrobial peptide that has the potential to be applied to a wide range of topical applications such as burns, wounds and skin infections. This market includes, but is not limited to, the mupirocin (Bactroban™; GSK) market. Mupirocin has no Gram-negative or fungal coverage and resistance is an ever-increasing problem, for example, mupirocin/methicillin-resistant *S. aureus* (MMRSA). HB-50 is now entering a number of preclinical trials exhibiting activity against multiple resistant *Pseudomonas*, *Staphylococcus* and *Candida* species. HB-64 is another broad-spectrum peptide that has shown, in a preliminary clinical trial, efficacy in reducing the severity of mild-to-moderate acne symptoms.

Four Helix peptides have demonstrated efficacy in the rat chronic lung infection model (reduction of 2 log orders in viable *P. aeruginosa* in 3 days). All lead candidates are active against multiple drug-resistant *P. aeruginosa* and specific peptides also demonstrate an anti-inflammatory effect. Once lead and back-up peptides have been identified, more rigorous preclinical testing including toxicology and inflammation end points are planned.

Certain peptides in the Helix library have been identified that have broad-spectrum activity against bacteria and yeast associated with sexually transmitted diseases and vaginitis. These pathogens include *Candida spp.* (including azole-resistant strains), *Haemaphilus ducreyei, Neisseria gonnorhoeae* and *Chlamydia trachomatis*. The lead peptides maintain their activity and cidality in gel formulation. Helix has advanced such peptides to preclinical trials.

4.8 Inimex Pharmaceuticals, Inc.

Inimex Pharmaceuticals, Inc. (Vancouver, BC, Canada) was founded in 2001 to further develop and commercialise new discoveries made by the laboratories of Bob Hancock and Brett Finlay at the University of British Columbia. This initiative involves the proprietary understanding of the functional genomics associated with the upregulation and control of the innate immune response. The company believes that modulating the innate immune response can provide potential new therapeutic strategies for the treatment of a number of diseases including bacterial infections, viral infections and cancer, as well as novel anti-inflammatory treatments. Inimex, based on peptides of the innate immune system, is currently screening for peptides and small molecules with the potential to selectively upregulate elements of innate immunity, while avoiding or limiting the damage caused by inflammation.

Inimex has demonstrated that its prospective lead peptide compounds can protect against bacterial infections in animal models. Inimex has also studied the mechanism of action for these novel peptides demonstrating that they induce an upregulation of genes determining chemokines and chemokine receptors, without stimulating the production of pro-inflammatory cytokines. In the past, attempts to utilise the immune response have resulted in a co-boosting of these inflammatory responses.

The main focus of Inimex's activities over the next 3 years will be on lead optimisation and investigational new drug (IND) studies. The company's initial product development will be based on current lead peptides delivered in combination with or in addition to existing antibiotic therapies for the treatment of nosocomial infections such as pneumonia. Subsequent development will focus on treatments for community-acquired infections and preventative medicines.

4.9 IntraBiotics

IntraBiotics (Mountain View, CA, USA) has concentrated its efforts on the topical antimicrobial market, building on technology initially licensed from Bob Lehrer's laboratory at University of California at Los Angeles (UCLA). Iseganan (IB-367) was developed by IntraBiotics based on the initial discovery of protegrins in porcine leukocytes [59]. It demonstrated an excellent *in vitro* profile exhibiting broad-spectrum antimicrobial activity, low resistance emergence and the maintenance of activity in saliva [60]. This activity translated well to the hamster cheek pouch model for oral mucositis in which the peptide significantly reduced oral bioburden. IntraBiotics went on to demonstrate safety and efficacy in Phase I and II clinical trials. However, a clinical benefit could not be achieved in Phase III trials in either radiotherapy or

chemotherapy patients. Iseganan HCl oral solution is in Phase II/III clinical trials for the prevention of ventilator-associated pneumonia (VAP). A second formulation, iseganan HCl solution for inhalation, has completed Phase I clinical trials in CF patients.

Since 2001 the company has significantly reduced its work force and focused on broadening its development candidates. The overall strategy of IntraBiotics is significant for two reasons. First, the company primarily developed iseganan for oral mucositis and then used the same peptide for further indications (line extensions). For a small company the consolidation of resources around one peptide saves time, resources and valuable money. However, this is at the expense of optimising a peptide's properties for clinical indications as diverse as oral bioburden reduction and the CF lung. Second, the company moved away from peptides in order to build a product development pipeline, which encompassed natural product drug discovery and development. This was done to allow the IntraBiotics to compete in the systemic antibiotic arena, configuring it as a pharmaceutical development company rather than an innate immunity peptide company.

4.10 Micrologix Biotech, Inc.

Micrologix Biotech, Inc. (Vancouver, BC, Canada) has developed clinical programmes based around intellectual property initially licensed from the Hancock Laboratory. These peptides, first isolated from bovine neutrophils by Michael Selsted's group [61], are primarily analogues of indolicidin. Micrologix has concentrated its efforts on topical applications including prevention of sepsis through reduction in central-line catheter contamination (MBI-226) and acne (MBI-594AN).

The company recently announced, in collaboration with Fujisawa Healthcare, Inc. (Deerfield, IL, USA), the completion of a Phase III clinical trial of MBI-226 for the prevention of central venous catheter-related bloodstream infections. The results did not show statistically significant superiority in preventing bloodstream infections, although the catheter colonisation rate was 31% in the MBI-226 group as compared with 40% in the providone iodine group ($p = 0.002$). This clinical end point certainly demonstrated the utility of such peptides in the clinical environment.

In September 2003, Micrologix announced the completion of a Phase IIb clinical trial of MBI-594AN in the treatment of acne. This is a 12-week study using a 2.5 or 1.25% gel formulation of the indolicidin analogue. This programme is advancing based on the encouraging results of the Phase IIa trial in which a significant reduction (> 40%) in inflammatory acne was observed in an alcohol-based product containing 2.5 or 5% peptide. No dose response was observed. The Phase IIb efficacy study confirmed the results. Statistically significant superiority was achieved by 2.5% MBI-594AN compared to vehicle control at 6 weeks in reducing inflammatory lesions ($p = 0.004$), non-inflammatory lesions ($p = 0.037$) and total lesions ($p < 0.001$). Micrologix is planning to take the product to a Phase III trial and FDA application in the future.

4.11 VDDI Pharmaceuticals

VDDI Pharmaceuticals (Brentwood, TN, USA) has concluded that local inactivation of peptide therapeutics at the site of action is a major contributor to the failure of certain peptides in clinical trials. However, such peptides have significant advantages: rapidly acting bactericidal effects; a unique mechanism of action; and there is strong evidence to support a unique and synergistic action when peptide and conventional antibiotics are used in combination. VDDI's strategy has been to apply its proprietary reverse peptide (REV-4) technology to medical peptides in order to overcome the inherent shortcomings of native compounds. Preclinical studies have confirmed the underlying concept of REV-4 technology by showing that multiple peptides selected from the magainin, cecropin, indolicidin and histatin families can be converted into their reverse forms while retaining activities against microorganisms relevant to human disease, including both bacteria and fungi. These peptides have also shown increased stability in biological matrices and reduced toxicity, and may therefore be suitable for systemic as well as topical applications.

The company's strategy is to identify sets of promising peptides, conduct the initial studies using REV-4 technology to establish proof of principle and then license the resulting compounds to pharmaceutical or biotechnology partners for further development. VDDI believes its unique approach has overcome many of the inherent shortcomings of first generation compounds and has thus positioned medical peptides as promising leads for a new generation of novel and highly effective antimicrobial compounds.

4.12 Xoma

Xoma (Berkley, CA, USA) has developed the product Neuprex™, an injectable formulation of $rBPI_{21}$, a modified recombinant fragment of BPI. BPI is a human host-defence protein made by polymorphonuclear (PMN) leukocytes. The native protein was discovered in 1978 by Elsbach and Weiss [44]. BPI kills Gram-negative bacteria, enhances the activity of antibiotics, neutralises Gram-negative endotoxin (a toxic molecule in the cell walls of Gram-negative bacteria that can trigger local and systemic inflammatory reactions in humans) and inhibits angiogenesis. These characteristics have led Xoma to develop various BPI derivatives, including $rBPI_{21}$, as well as several smaller peptide-sized derivatives. $rBPI_{21}$ has been proven to be safe in Phase I human trials, shown promise in Phase II trials and has recently completed a Phase III trial for severe meningococcaemia, but has only demonstrated a trend towards an apparent benefit. Xoma has an ongoing collaboration with the Joslin Diabetes Center (an affiliate of the Harvard Medical School), investigating rBPI-derived compounds in models of retinopathies. Diabetic retinopathy is the most common cause of blindness in adults. In diabetics, chronic

high blood glucose levels trigger neovascularisation (abnormal growth of blood vessels in the retina). Macular degeneration, another major cause of adult blindness, also involves angiogenesis of retinal blood vessels. Retinopathy of prematurity is a potentially blinding angiogenic condition that affects premature infants. The company is also evaluating a topical antibacterial formulation of a BPI-derived compound for the potential treatment of acne and plans to initiate clinical testing in late 2003.

In 2000, Xoma licensed certain rights to the Neuprex formulation to Baxter Healthcare Corporation, but in July 2003, Xoma regained all rights. Xoma is seeking a pharmaceutical partner to advance development of Neuprex in multiple anti-infective and antiendotoxin indications.

4.13 Zengen

Zengen (Woodlands, CA, USA) is developing peptide molecules derived from α-melanocyte-stimulating hormone (α-MSH). One such peptide, CZEN-002, has been the focus of the company's research into the control of infection. This synthetic octapeptide was developed using > 2 5years of original research in the US, Europe and Asia resulting in evidence of anti-inflammatory and anti-infective activity from both *in vivo* and *in vitro* research. In preclinical studies, CZEN-002 has been shown to directly kill pathogenic fungi and Gram-negative and -positive bacteria and to inhibit replication of HIV-1. Zengen is currently conducting Phase I/II clinical trials with CZEN-002 in vaginitis.

5. Expert opinion

The development of cationic antimicrobial peptides into viable clinical therapeutics has, to some degree, become segmented into three approaches. First, topical application. This is still undoubtedly the most accessible approach for peptide drugs, mainly due to the properties of such peptides in nature. There are companies that have concentrated upon the reduction of peptide size and cost with the intention of applying such therapeutics topically (Helix BioMedix, Micrologix Biotech and Zengen). Lessons learned in the past include the fact that choice of topical application needs to be selected with care to avoid indications in complex patient populations with difficult-to-define end points. For example, the application of innate immunity peptides to oral mucositis would seem to be a very good fit based upon the advantages of the class; microbicidality, low resistance threat, low systemic absorption, activity in saliva and *in vivo* reduction in oral bioburden. In addition, oral mucositis is an unmet medical need in chemotherapy patients, which currently has no approved therapy. However, the extent and precise role of bacteria and fungi in the pathogenesis of this condition and the effectiveness of antimicrobial agents in this complex patient population with multiple clinical manifestations is still unknown [62,63]. The same issues may have contributed to the failure of Magainin Pharmaceuticals (Genaera Corporation) to gain FDA approval for their lead peptide, pexiganan, used for infections of diabetic foot ulcers. In the past the temptation has been to pursue such indications in high-cost patient populations, due to the relatively high cost of certain peptides.

The development of high-cost peptides for topical applications is not realistic. If peptide medication cost, through design and delivery, can be reduced then simpler and more realistic applications with more clearly defined end points can be explored. Such areas tend to mimic those in which peptides of the innate immune system operate (e.g., the protection of mucosal surfaces). It should also be noted that the direct application of topical therapeutics raises fewer issues of toxicity, requires less drug substance and enables a relatively high concentration of active compound to be delivered, reducing the likelihood of the emergence of resistance.

The second approach, also within the topical area, is the utilisation of the more recently discovered immunomodulatory functions of innate immunity peptides (Inimex, Helix BioMedix and Zengen) for the treatment of infectious disease and inflammatory conditions. A viable option for companies working in this field is that of niche applications that are primarily topical. However, such areas should also be selected with care to avoid the same complexity described for topical applications in general, such as complex patient populations with difficult-to-define end points.

Third, although the development and marketing paths for systemic antimicrobial agents are difficult at present, a number of peptide companies are exploring this area (Adaptive Therapeutics, VDDI, AM Pharma, Entomed and Xoma) with the application of larger recombinant peptides, 'reverse-peptides' or cyclised sequences containing D-amino acids. The selective pressure of widespread resistance on the pharmaceutical and investment community is not yet a strong enough commercial driver for the development of an antibiotic active against multi-resistant organisms. The market size for a drug of last resort seems limited particularly in the treatment of Gram-positive infections. In addition, the returns on development for such products are relatively low compared to other existing and emerging therapeutic areas. These problems have prompted some 'large pharma', potential development partners for small biotechnology companies, to exit the field. To overcome these obstacles there will need to be true innovation in the antimicrobial peptide field with respect to systemic therapeutics. Whether or not the technology currently being developed, described above, provides that innovation will be determined over the next few years.

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ABSTRACTS

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Skin mast cells provide defense against invasive bacterial infection

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Mast cells (MC) express cathelicidins (Caths), peptides that act as antibiotics by killing microbes. We hypothesized that presence of MC, and their expression of Cath, will influence skin infections. To evaluate this, MC-deficient mice (c-Kit wsash KO) were compared to normal controls for resistance to invasive Group A Streptococcus (GAS). Compared to controls, C-Kit wsash KO had 30% larger lesions, 80% more lesional bacteria and 30% more bacteria in the blood 4 days after subcutaneous (sc) injection with GAS. To determine if these differences were due to the presence of MC, and determine the relative role of Caths, we reconstituted the skin of c-Kit wsash KO with MC derived from the bone marrow of either wild type or Cath deficient mice (Cnlp-/-), and challenged them with GAS, 2 weeks after MC transplant. At 48 hours after GAS injection mice that did not receive MC had an average lesion size of 250sqmm. Mice reconstituted with wild type MC showed lesions comparable with normal (10sqmm) while the mice reconstituted with Cath deficient MC showed a lesion size of 200sqmm. This Cath-dependent susceptibility to infection also resulted in greater systemic bacteremia as spleens recovered from these mice showed an increase in bacterial load. To better define the role of MC in controlling invasion, we designed a model in which bacterial entry is from the surface rather than injected. GAS bacteria were grown at 2.1*10ES/ml into a THB gel and applied for 18 hours to the surface of either C-Kit wsash KO mice or normal littermates. The gel was removed and skin washed and sampled to measure bacteria. In contrast to the data obtained when the GAS was injected sc, no significant differences were noticed between groups, indicating that MC activity is after barrier penetration. On the other hand, mice that lack Cath in keratinocytes show increased topical GAS survival. These results show for the first time that MCs in the skin provide defense against bacterial infection, a function mediated in part by the expression of cathelicidin antimicrobial peptides.

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Chitosan nanoparticles: Agent for topical treatment of cutaneous inflammatory and infectious diseases

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Nanoparticles have thus far demonstrated excellent medicinal controlled delivery and stabilization. Using nanoparticles which are nontoxic, biodegradable, and biocompatible as the actual therapeutic is the ideal next step. We developed and investigated the potential of chitosan nanoparticles as an anti-inflammatory agent, as demonstrated by the suppression of P. acnes induced inflammatory cytokine IL-12p40. We further demonstrated that not only do chitosan nanoparticles have anti-inflammatory properties, but also antimicrobial action. CFU assays of P. acnes, E. coli, and S. aureus treated with these nanoparticles demonstrated effective killing impact with increasing concentrations. We evaluated the mechanism of bactericidal killing of the nanoparticles via SEM/TEM imaging, which demonstrated that chitosan nanoparticles induced disruption of the bacterial cell membrane. Together, these data suggest that chitosan nanoparticles could be used for both inflammatory and infectious dermatological conditions.

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Pimecrolimus enhances innate immune function of normal human keratinocytes

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Recent studies suggest that innate and adaptive immunity are controlled by distinct and sometimes contrasting mechanisms. Calcineurin inhibitors inhibit elements of the adaptive immune system, i.e. T-cell activation and cytokine release, and are effective drugs for inhibition of inflammation in the skin. However, the effects of calcineurin inhibitors on innate immunity are unknown. Therefore, we asked if the calcineurin inhibitor pimecrolimus affects the innate immune response of the epidermis by assessing the ability of cultured human keratinocytes (NHEK) to kill bacteria, express Toll-like receptors (TLRs), and produce antimicrobial peptides (AMPs). In contrast to its immunosuppressive effects on adaptive immune responses, pimecrolimus (10nM) increased the ability of NHEK to kill Staphylococcus aureus mprF in culture ($p<0.05$), and stimulated an increase in TLR1 (320%, $p<0.05$) TLR2 (190%, $p<0.05$), and TLR6 (440%, $p<0.05$) as measured by qPCR. In response to the TLR2/6 ligand Malp-2, and the TLR2/1 ligand LTA, pimecrolimus also increased NHEK expression of cathelicidin AMP mRNA (890%, $p<0.01$ and 530%, $p<0.05$) and enhanced immunostaining for the peptide. The induction of cathelicidin mRNA by pimecrolimus and Malp-2 was further enhanced by 250% with the addition of 1nM 1,25 vitamin D3. Furthermore, pimecrolimus increased human beta defensin-2 (HBD2) and HBD3 by 740% ($p<0.05$) and 800% ($p<0.01$) each over Malp-2 alone. The response to other TLR ligands remained unchanged. In conclusion, pimecrolimus surprisingly enhanced the innate immune function of keratinocytes, demonstrating differential effects on innate immune and adaptive immune responses. Further studies are needed to validate these findings in vivo, and may possibly provide a novel explanation for the clinical effects of this drug.

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Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D dependent mechanism

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The innate immune response to injury or infection relies on the capacity to recognize microbes and stimulate production of antimicrobial peptides (AMP). In this study we investigated how Toll-like-receptors (TLR) and AMP are controlled by injury. Keratinocytes surrounding a 24 hr aseptic human wound increased expression of CD14 (8-fold) and TLR2 (5-fold) by qPCR, accompanying a large and previously known increase in cathelicidin AMP (hCAP18). hCAP18 and CD14 were known to be induced by 1,25OH vitamin D3 (1,25D3), and analysis of TLR2 expression revealed this also was induced by 1,25D3. Topical 1,25D3 application to human skin confirmed these results, showing increased hCAP18 and TLR2 by immunostaining. Furthermore, 1,25D3 enabled keratinocytes to respond to Malp2 (a TLR2/6 ligand) with increased cathelicidin production which was inhibited by neutralizing antibody to TLR2. 1,25D3 also increased the ability of keratinocytes to kill S. aureus. Thus, we hypothesized that 1,25D3 was a signaling molecule during injury. Supporting this, we found that CYP27B1, the enzyme that converts 25D3 to active 1,25D3, was increased 4-fold in wounds and induced in response to TGFβ1 (8-fold) or Malp2 (4-fold). Blocking the vitamin D receptor, inhibiting CYP27B1 enzymatic activity, or limiting 25D3 in culture each prevented TGFβ1 from inducing hCAP18, CD14 or TLR2. Furthermore, mice deficient in CYP27B1 failed to increase CD14 in vivo following injury. Thus, this investigation demonstrates how injury initiates the innate immune response; vitamin D3 is activated by enzymatic conversion, a process triggered by microbial products or host factors such as TGFβ1. The increase in 1,25D3 then directly increases cathelicidin release and enables responsiveness to microbial products through induction of TLR2 and CD14.

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IL-1β and the inflammasome but not IL-1α mediate IL-1R-dependent neutrophil recruitment against *Staphylococcus aureus* skin infection

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We previously reported that activation of IL-1R-signaling by resident skin cells is critical for neutrophil recruitment and host defense against S. aureus skin infections. Here, the differential contribution of IL-1α, IL-1β, and the inflammasome in mediating IL-1R-dependent neutrophil recruitment against S. aureus skin infection was investigated using a mouse model of cutaneous infection with S. aureus in conjunction with bioluminescent bacteria to track bacterial growth. We found that skin lesions of S. aureus-infected IL-1β- and IL-1α/β-deficient mice were substantially larger (3-4 fold) with higher bacterial counts (10-15 fold) and had a severe defect in neutrophil recruitment and in myeloperoxidase activity (2 fold) compared with wildtype control mice or IL-1α-deficient mice ($p<0.05$). This severe phenotype of skin lesions of S. aureus-infected IL-1β- and IL-1α/β-deficient mice closely resembled skin lesions of S. aureus-infected IL-1R-deficient mice. Thus, IL-1β and not IL-1α mediates IL-1R-dependent neutrophil recruitment and host defense against S. aureus skin infections. Furthermore, mice deficient in ASC, an inflammasome component that associates with caspase-1 to promote conversion of pro-IL-1β into active IL-1β, had a similar severe phenotype of skin lesions as IL-1β-deficient mice after S. aureus skin infection. Taken together, these data provide evidence that effective neutrophil recruitment and bacterial clearance of S. aureus skin infections is dependent upon inflammasome activation and generation of IL-1β, which subsequently activates IL-1R-signaling to promote neutrophil recruitment to a site of S. aureus infection in the skin.

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Methicillin-resistant *staphylococcus aureus* colonization in children with atopic dermatitis

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Children with atopic dermatitis (AD) are more frequently colonized with Staphylococcus aureus (SA) than children without AD. However, little data exist regarding the prevalence of methicillin-resistant SA (MRSA) among children with AD. An increasing prevalence of community-associated MRSA (CA-MRSA) has been seen among those presenting to hospitals with serious bacterial infections. Since many AD patients are treated empirically with antibiotics for secondary skin infections, an understanding of the epidemiology of bacterial colonization and superinfection is essential for directing proper treatment in the atopic patient population. An observational cross-sectional study was conducted at the Children's Hospital of Philadelphia in which 54 patients previously diagnosed with AD were enrolled. A detailed patient questionnaire, a complete cutaneous examination, and an evaluation of eczema severity according to the Eczema Area and Severity Index (EASI) were completed at enrollment. Bacterial cultures from the skin and nares were obtained to determine the frequency of colonization with either methicillin-sensitive SA (MSSA) or MRSA. Although most AD patients studied were colonized with SA [43/54 (80%)], MRSA was isolated from only 7 AD patients [7/54 (13%)]. Patients colonized with SA were more likely to be male, to have been previously hospitalized, to have used a topical calcineurin inhibitor in combination with a topical steroid, and less likely to have used topical antibiotics. Bivariable analysis, however, revealed that only previous hospitalization was independently associated with an increased risk of MRSA colonization.

Macrolactam immunomodulators for topical treatment of inflammatory skin diseases

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The immunomodulatory macrolactams provide an alternative to glucocorticosteroids for the topical treatment of atopic dermatitis and other inflammatory dermatoses. Tacrolimus (FK506), as well as the newer ascomycin derivative ASM 981 (pimecrolimus), penetrate the inflamed epidermis and are suitable for topical therapy. Both substances inhibit the transcription of proinflammatory cytokine genes such as interleukin 2, which are dependent on the nuclear factor NF-AT. They block the catalytic function of calcineurin, which leads to the inhibition of the transport of the cytoplasmic component of NF-AT to the cell nucleus. Multicenter, randomized, double-blind clinical trials with topical formulations have shown the efficacy of both substances in moderate to severe atopic dermatitis. A review is presented of the biochemical and cell biologic properties, mode of action, pharmacokinetic data, side effects, results of the clinical trials, and further indications for tacrolimus and ASM 981, along with an overview of the related substances cyclosporine and sirolimus (rapamycin). (J Am Acad Dermatol 2001;45:736-43.)

Patients with chronic inflammatory skin diseases, particularly those with atopic dermatitis and psoriasis, suffer from a markedly impaired quality of life and present many therapeutic challenges. Although the mainstay of successful long-term treatment of both disorders is the identification and elimination of triggering factors in combination with an appropriate atopic dermatitis skin maintenance program using emollients, almost every patient occasionally requires more intensive therapy.[1] During disease flares, it is useful to administer anti-inflammatory drugs, which alter the immunologic mechanisms involved in the pathogenesis of the disorders.[2,3] Although many drugs are available for the systemic treatment of these diseases, all have inconvenient features or side effects that limit their use. Possible agents include methotrexate, the use of which is limited because of hepatotoxicity; cyclosporine, which may lead to hypertension and nephrotoxicity; mycophenolate mofetil, which may induce anemia; retinoids, which bear the risk of teratogenicity; and various UV light regimens such as PUVA, which is associated with an increased risk of skin cancer. The most important acute adverse effect when treating inflammatory skin diseases with systemic immunosuppressants is nonspecific immunosuppression, potentially leading to cutaneous and extracutaneous infections. Because patients with atopic dermatitis are already at higher risk for the development of cutaneous and extracutaneous infections, systemic therapy should be restricted to patients with severe disease.

Because topical application of drugs frequently reduces the unwanted effects of systemic administration, topical formulations are generally preferred. The ideal drug for the treatment of chronic inflammatory skin diseases should therefore be topical, highly effective, simple to use, cosmetically acceptable, and with as few adverse effects as possible. The currently available agents fail to even approach these goals. Coal tar and anthralin have been almost completely abandoned for cosmetic reasons and because of potential carcinogenicity. Topical glucocorticoids were introduced about 5 decades ago and have radically improved treatment. More recently developed forms, such as the nonhalogenated double esters, show an even better risk-benefit ratio than their predecessors.[4] However, there are significant potential side effects associated with topical corticosteroids, including skin atrophy, hirsutism, and even systemic absorption. Fear of these problems has led to widespread and often illogical public mistrust of topical corticosteroids in Europe.

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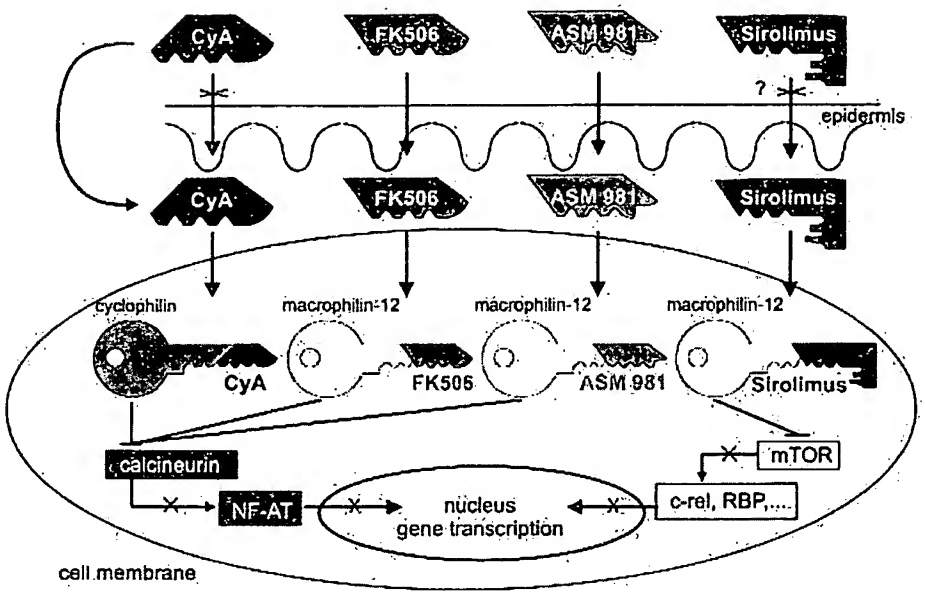

Fig 1. Mechanism of action of macrolactam immunomodulators. *CyA,* Cyclosporine; *mTOR,* mammalian targets of rapamycin; *NF-AT,* nuclear factor of activated T cells; *RBP,* rapamycin binding protein.

Therefore other substance groups have been investigated as alternatives for topical treatment, including calcineurin inhibitors, a group of immunosuppressive drugs that is well established and frequently used in transplantation medicine. The T-cell response to protein antigens critically depends on the induction of several proinflammatory cytokines controlled by a nuclear factor called nuclear factor of activated T cells (NF-AT); it also depends on the presence of certain membrane proteins. In a variety of ways, calcineurin inhibitors alter this pathway. Their topical use may be an effective treatment of inflammatory skin diseases, while restricting the unwanted effects to the skin.

Cyclosporine has the longest history of systemic use, but has poor skin penetration.[5-7] Other immunomodulatory substances that share a macrolactam structure and a potential for topical use are referred to as topical macrolactam immunomodulators.[8] Tacrolimus (FK506), regarded as the key substance of the macrolactam immunomodulators, has been shown to be highly effective topically, especially in atopic dermatitis, but also in a growing list of other skin diseases.[9] A newer macrolactam immunomodulator, the ascomycin derivative ASM 981, also shows promising results,[10] whereas the role of sirolimus remains to be determined.[11] We review current knowledge about these substances, covering the mechanisms of action (Fig 1), pharmacokinetics, clinical efficacy, and side effects, concentrating on the dermatologic aspects of topical use.

PHYSICOCHEMICAL CHARACTERISTICS AND EXPERIMENTAL DATA

Cyclosporine

Cyclosporine was isolated from the fungus *Tolypocladium inflatum* and became the first clinically useful T-cell active immunosuppressive agent. Since its introduction in the early 1980s, cyclosporine has revolutionized immunosuppressive therapy in transplantation medicine.[12] It is a highly lipophilic cyclic polypeptide with a molecular weight of 1202 D, which binds to an intracellular receptor, the immunophilin cyclophilin.[7] The cyclosporine-cyclophilin complex inhibits the calcium-dependent serine-threonine-phosphatase calcineurin. Calcineurin is responsible for the activation of the nuclear factor of activated T cells (NF-ATp), which itself activates, among others, the transcription of interleukin 2 (IL-2).[13,14] Thus cyclosporine suppresses the production of IL-2, resulting in the inhibition of proliferation and activation of T cells, thereby reducing the T-cell–mediated immune response.[14] Cyclosporine has many other immunosuppressive actions, including the inhibition of secretion of several other NF-ATp-dependent cytokines. Thus cyclosporine belongs to the functional substance group of calcineurin inhibitors, but is not a member of the structurally and functionally defined group of macrolactam immunomodulators. Because cyclosporine is generally unsuitable for topical use in clinical dermatology,[7] it is discussed briefly throughout this article for mostly historical and didactical reasons.

Tacrolimus

Tacrolimus (FK506) was first isolated in 1984 from the culture broth of a *Streptomyces* species found in Tsukuba, Japan. The name "tacrolimus" is a neologism, composed of Tsukuba macrolide and immunosuppressant. The first report on this new drug was published in 1987.[15] The newly identified fungus was named *Streptomyces tsukubaensis* after its origin and is characterized by a gray mycelium, spore chain with smooth spore surfaces, nonchromogenicity, and limited carbohydrate usage.[16]

Although tacrolimus shows similar biologic properties to cyclosporine in vivo and in vitro, there is no structural relationship. Tacrolimus is a macrolide lactone and in its natural form is a white powder with an atomic weight of 822 D.[16] Because of its hydrophobicity, tacrolimus is virtually insoluble in water but may easily be dissolved in methanol, chloroform, acetone, or ethanol. Similar to cyclosporine, tacrolimus inhibits the activation and maturation of T cells and blocks transcriptional activation of several lymphokine genes. Tacrolimus exerts its biologic effects after binding to cytosolic proteins, the macrophilins, formerly called FK506-binding proteins (FK-BP).[17,18] These proteins are immunophilins, just like cyclophilin. The complex of tacrolimus and macrophilin 12 blocks calcineurin, inhibits the transcription of NF-ATp-dependent genes,[19] inhibits IL-2 transcription, and blocks the T-cell response.[14] Other targets of tacrolimus include the NF-ATp-dependent cytokines IL-4 and IL-5.[20] Treated keratinocytes down-regulate IL-8 receptors on their cell surface.[21] The expression of functionally relevant surface molecules on Langerhans cells is altered, perhaps causing a decrease in their allostimulatory capacity.[22] The high-affinity IgE receptor (FcεRI), present on Langerhans cells and inflammatory dendritic epidermal cells in lesional skin of atopic dermatitis,[23] is also down-regulated with topical tacrolimus treatment and the number of inflammatory dendritic epidermal cells falls below the detection limit.[24] Furthermore, the antigen-presenting capacity of lesional epidermal cell suspensions toward autologous lymphocytes is reduced.[24] Incubation of anti-IgE-activated skin mast cells and basophils with tacrolimus leads to the inhibition of histamine release.[25] Although the role of mast cells in atopic dermatitis is not clear at present,[26] these data might explain the unusually rapid clearing of atopic dermatitis after topical tacrolimus therapy.[27] Animal studies with an induced allergic contact dermatitis model in mice showed reduced RNA levels of IL-1α, IL-1β, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α, macrophage inflammatory protein 2, and interferon gamma from tacrolimus.[28] As compared with cyclosporine, about 100 times lower concentrations of tacrolimus are effective in vitro and in vivo.[16]

ASM 981

The ascomycin derivative ASM 981, also known as SDZ ASM 981 or oversimplified as "ascomycin," is a macrolide with a molecular weight of 810 D. The generic name pimecrolimus has just recently been assigned. True ascomycin, the parent compound, was originally isolated in the early 1960s from the fermentation product of *Streptomyces hygroscopicus* var *ascomyceticus* and showed antifungal activity.[29] Two derivatives, SDZ 281-240 and SDZ ASM 981, are effective topically both in animal models of cutaneous inflammation and in inflammatory skin diseases in humans.[30] Many experimental and clinical studies on topical use of ASM 981 have been published,[18] whereas few data, none recent, are available on the apparently abandoned SDZ 281-240 component.

Because ASM 981 also interacts with macrophilin-12, its clinical effects closely resemble those of tacrolimus. They seem to differ in therapeutic effectiveness as well as in their structure-related limitations of formulation. ASM 981 interferes with stimulation of T cells by antigen-presenting cells, blocking both helper T cell type 1 (T_H1) cytokines such as IL-2 and interferon gamma and T_H2 cytokines such as IL-4 and IL-10.[31] In mast cells, liberation of mediator substances such as hexosaminidase, tryptase, and histamine is inhibited as well as transcription of the late-phase cytokine tumor necrosis factor α.[18,31] Studies with induced allergic contact dermatitis in mice, rats, and pigs demonstrated the anti-inflammatory effects of ASM 981.[32] Its topical activity in a pig model was comparable with the highly potent glucocorticoid clobetasol-17-propionate.[10] No skin atrophy was observed in the animals after ASM 981 application, in contrast to the well-established effects of potent glucocorticoids.[10]

Sirolimus

Sirolimus is another macrolactam immunomodulator, with a molecular weight of 914 D. It has formerly been called rapamycin because the producing fungus was isolated from a soil sample brought from Rapa Nui (Easter Islands) in 1965.[33] Like tacrolimus, it binds to macrophilin-12 and other immunophilins. The biologic effects of the sirolimus-macrophilin-12 complex are different from those of other drug-macrophilin complexes already discussed. The target structures of the sirolimus-macrophilin-12 complex are a group of proteins named "mammalian targets of rapamycin,"[34] also known as FK-BP-rapamycin-associated protein,[35] "sirolimus effector proteins," or "RAP

targets."[34] These proteins are highly conserved in eukaryotes and contain a kinase-like domain in the C-terminal 400 amino acids, which exhibits 25% protein identity with the kinase domain of phosphatidylinositol-3-kinase.[34] This family of kinases is believed to be involved in a broad range of physiologic processes linked to control of the cell cycle. Sirolimus seems to affect the cell cycle of the activated cell at the G1 to S phase, whereas cyclosporine, tacrolimus, and ASM 981 block the cell cycle in an earlier phase, G0/G1. Interference with a Ras-independent signal transduction pathway, required for the activation of the 70-kd S6 protein kinase ($p70^{sk6}$), might explain the growth-suppressive effect of rapamycin on lymphoid cells.[36,37] Keratinocyte stem cells treated with sirolimus exhibited a decreased proliferating cell nuclear antigen expression, whereas cyclin D1 expression, which precedes proliferating cell nuclear antigen expression in the cell cycle, was not affected.[38]

The immunosuppressive qualities of topical sirolimus application have been studied in different animal models with contradictory results in vitro and in vivo. Beneficial effects of sirolimus in phorbolester-induced ear swelling were shown in 1992.[32] However, topical application of 1.2% sirolimus in a pig skin model of allergic contact dermatitis was ineffective.[39] Furthermore, topical application of up to 2% sirolimus ointment was ineffective in a guinea-pig contact dermatitis model, although suppression of keratinocyte proliferation was observed.[40]

SYSTEMIC USE: CLINICAL EFFICACY AND SIDE EFFECTS

Cyclosporine

Cyclosporine was the first T-cell active immunosuppressant approved for the systemic treatment of severe psoriasis and atopic dermatitis. In addition, a variety of other inflammatory skin diseases are also responsive.[7,41] The majority of data concerning efficacy, long-term safety, and adverse effects of the calcineurin inhibitors refer to cyclosporine. Although its systemic use is effective, especially in atopic dermatitis and psoriasis, predictable adverse effects such as hypertension and nephrotoxicity limit its systemic use.[7,12] Because of the immunomodulatory effects of cyclosporine, there is also an inhibition of tumor defense mechanisms, as supported by a higher incidence of skin cancers in cyclosporine-treated organ transplant patients.[42] There are fewer data on dermatologic patients on a low-dose regimen, but the incidence of lymphoma is less than 0.2%.[12] In addition to the immunosuppression, there appears to be a transforming growth factor β–mediated autonomous mechanism of cyclosporine-induced carcinogenesis,[43] but the clinical relevance of this effect remains to be determined. These adverse effects help explain why, although highly effective, systemic cyclosporine should be restricted to patients with severe inflammatory skin diseases only.[12]

Tacrolimus

Oral tacrolimus is licensed for the prophylaxis and treatment of rejection after kidney or liver transplantation in many countries. The first report of systemic tacrolimus in dermatology in 1992 described clearing of psoriasis in 4 patients.[44] The clinical efficacy in psoriasis has been confirmed by a European multicenter double-blind placebo-controlled clinical trial, using a daily dosage of 0.05 to 0.15 mg/kg.[45] Similar efficacy is anticipated for the treatment of atopic eczema. The efficacy of oral tacrolimus in the treatment of alopecia areata,[46] Sézary syndrome,[47] and pyoderma gangrenosum[48] has been described in case reports; the dosages ranged from 0.1 to 1 mg/kg per day. Because oral tacrolimus is not licensed for skin diseases in Europe, its use should be restricted at this time to controlled clinical trials, but it may become an alternative to cyclosporine in treatment of severe inflammatory skin diseases.

As with cyclosporine, numerous adverse effects limit the systemic use of tacrolimus. Hypertension, electrolyte disturbances, neurotoxicity, nephrotoxicity, and altered glucose metabolism have been reported as side effects. Women taking tacrolimus after transplantation do well during pregnancy, with a surprisingly low incidence of hypertension, preeclampsia, and other maternal complications.[49] In contrast to cyclosporine, hypertrichosis has not been reported in patients treated with tacrolimus.[46] Whereas oral tacrolimus is metabolized in liver and gut, topical tacrolimus is almost exclusively metabolized in the liver by the cytochrome P-450 IIIA4 isoenzyme,[9] thus explaining the known drug interactions with other substances (eg, erythromycin, known to be metabolized by the P-450 IIIA4 isoenzyme).[50]

ASM 981

ASM 981 is said to be developed specifically for topical use. A double-blind, placebo-controlled, randomized clinical trial is currently being performed to evaluate the efficacy of oral ASM 981 in severe chronic plaque psoriasis,[51] but the study is still blinded and no final data are available at present.

Sirolimus

In October 1999, an oral preparation of sirolimus was licensed in the United States to prevent kidney rejection in transplantation medicine. There may be a role for oral sirolimus in the treatment of psoriasis, but no clinical phase II study has been published. The

direct effect of systemic sirolimus treatment on keratinocyte stem cell proliferation was investigated in vitro and in vivo in patients with psoriasis, who received oral sirolimus, 5 mg/m^2 per day, for 1 week.[38] As shown in patients with severe psoriasis, systemic administration of sirolimus may lead to fever, anemia, and capillary leak syndrome,[52] which restricts the potential use of this drug. These severe adverse effects may be a result of drug-induced apoptosis of lesional leukocytes, especially activated T cells, and possibly release of inflammatory mediators.[52]

TOPICAL USE IN INFLAMMATORY SKIN DISEASES

Cyclosporine

Topical application of cyclosporine does not lead to therapeutic levels of cyclosporine in the skin, unless skin penetration is facilitated by addition of penetration enhancers such as a polyarginine linker.[53] Clinical trials with topical cyclosporine preparations have been performed but did not show satisfactory results.[5,6] This lack of topical efficacy seems to be due to a limited penetration of cyclosporine through the epidermis, which may be explained by the size of the molecule.[54] Consequently, the ulcerative lesions of pyoderma gangrenosum with a defective epidermal barrier are the only clinical setting that responds well to topical cyclosporine.[55]

Tacrolimus

The penetration of topical tacrolimus is highly variable and depends on the concentration, vehicle, and integrity of the epidermal barrier. Its penetration is best in inflamed skin, compared with normal skin, which explains its high efficacy on lesions compared with its minimal effects on normal skin. Tacrolimus ointment reproducibly achieves therapeutic concentrations in skin affected by atopic dermatitis.[56-58] The first large European placebo-controlled, double-blind, clinical multicenter trial involving 213 patients with atopic dermatitis confirmed the efficacy of topical tacrolimus for this disease.[56] These results, which included rapid alleviation of pruritus and lesions and improvement of scores, were soon confirmed by a phase I study undertaken with 0.3% tacrolimus ointment in the United States.[59] An accumulation of the topically applied drug could be excluded in both trials. Another randomized, placebo-controlled, double-blind, multicenter trial proved its safety and efficacy in children aged 7 to 16 years.[57] Allergic contact dermatitis has been shown to respond to topical tacrolimus.[50] In psoriasis, as compared with atopic dermatitis, topical tacrolimus seems less effective. A 6-week pilot study with nonoccluded 0.3% tacrolimus ointment for chronic plaque psoriasis showed little change,[60] most probably because of unsatisfactory penetration of the epidermis. Case reports suggest that topical tacrolimus may work in patients with pyoderma gangrenosum[61] and oral lichen planus.[62] Tacrolimus ointment was licensed in Japan in 1999 for the treatment of atopic dermatitis and was launched in the United States in 2001.

The most frequently observed side effect of short-term treatment is a transient burning or heat sensation, which starts a few minutes after application of the ointment and lasts for about 30 to 90 minutes.[56] Duration and intensity of this burning are maximal after the first application and diminish during the next 5 to 10 days. If the therapy is discontinued, another period of burning must be expected if it is restarted. Physical examination, measurement of skin thickness with ultrasound, and biochemical studies of the collagen metabolism in a randomized, double-blind, placebo-controlled trial clearly showed that topical tacrolimus does not interfere with collagen synthesis and does not lead to skin atrophy.[63] An open-label, noncomparative, multicenter phase III study assessed the long-term safety of 0.1% tacrolimus ointment in 316 adult patients with atopic dermatitis.[58] The long-term safety profile of 12 months of treatment was similar to that of short-term trials, confirmed the lack of skin atrophy, and reflected complications generally associated with atopic dermatitis.[58] These data have recently been confirmed by an independent clinical trial involving a total of 255 children, 2 to 15 years of age, for up to 12 months.[64] Meanwhile more than 10,000 study patients have been treated worldwide with topical tacrolimus. More prolonged adverse effects such as possible carcinogenicity remain to be monitored.

ASM 981

The efficacy of topical ASM 981 in patients with atopic dermatitis was shown in 1998 in a randomized, double-blind, placebo-controlled trial.[65] In 34 patients a 1% ASM 981 ointment proved to be significantly superior to placebo, and no clinically relevant adverse events were reported. Subsequently, studies have been undertaken in 1- to 4-year-old children[66] and 5- to 16-year-old children[67] with atopic dermatitis, demonstrating relatively low systemic drug levels in those children treated with 1% ASM 981 cream. A placebo-controlled, double-blind, clinical trial involving 10 patients confirmed that chronic psoriatic plaque lesions may respond to ASM 981 under the occlusive conditions of a microplaque assay.[41] Long-term safety and efficacy studies with ASM 981 have not yet been published. Approximately 2000 patients have been treated.

Sirolimus

No clinical data about humans treated with topical sirolimus preparations are available at this time.

PERSPECTIVES

The best established current indications for oral therapy with T-cell-active immunomodulatory agents in dermatology are severe cases of psoriasis vulgaris and atopic dermatitis. In Europe, cyclosporine is the only drug in this category of substances currently licensed for systemic treatment of skin diseases. The systemic use of T-cell-dependent immunomodulatory drugs will likely remain limited to patients with a severe and otherwise difficult to control disease course. The range of potential long-term side effects may be even more limiting than the cost of treatment.

In view of the much more favorable relation between therapeutic and adverse effects, the topical use of macrolactam immunomodulators will probably increase over the next several years. These drugs might represent an alternative to glucocorticoids in the treatment of chronic inflammatory skin diseases, especially atopic dermatitis.[2,9,41,68] Both case reports and large, multicenter, randomized, controlled, double-blind clinical trials have shown the efficacy and safety of tacrolimus. There are fewer published corresponding data on ASM 981, but many promising results have recently been presented in clinical meetings. Both drugs appear to be a promising alternative to corticosteroids, whereas sirolimus still needs to prove its efficacy in a clinical setting. Even less is known about the dunaimycins, a new complex of spiroketal 24-membered macrolides discovered in the fermentation broth of two *Streptomyces diastatochromogenes* strains, which are known to possess both immunosuppressive and antimicrobial activity.[69-71]

A lack of epidermal atrophy and striae distensae, both potential side effects of topical glucocorticoid therapy, has been demonstrated for both tacrolimus and ASM 981[63,72] and suggests that the macrolactam immunomodulators are well suited for treating chronic inflammatory skin diseases. There are no data suggesting clinically relevant long-term side effects. Nevertheless, the risk of these potential side effects such as increased carcinogenesis and especially photocarcinogenesis should be monitored carefully. In the future, alopecia areata, lupus erythematosus, lichen planus, and pyoderma gangrenosum might become additional indications for topical therapy with these novel macrolactam immunomodulators.

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REVIEW ARTICLE

Pimecrolimus: A review

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ABSTRACT

Pimecrolimus (SDZ ASM 981), an ascomycin derivative, is one of the new classes of immunomodulating macrolactams and was specifically developed for the treatment of inflammatory skin diseases. The interest in pimecrolimus has been substantial because of its significant anti-inflammatory activity and immunomodulatory capabilities and its low systemic immunosuppressive potential. The mechanism of action of pimecrolimus is the blockage of T cell activation. Pimecrolimus (like all ascomycins) is an immunophilin ligand, which binds specifically to the cytosolic receptor, immunophilin macrophilin-12. This pimecrolimus-macrophilin complex effectively inhibits the protein phosphatase calcineurin, by preventing calcineurin from dephosphorylating the nuclear factor of activated T cells (NF-AT), a transcription factor. This results in the blockage of signal transduction pathways in T cells and the inhibition of the synthesis of inflammatory cytokines, specifically Th1- and Th2-type cytokines. Pimecrolimus has also been shown to prevent the release of cytokines and pro-inflammatory mediators from mast cells. Several studies have evaluated the effectiveness of pimecrolimus as a treatment for skin diseases. In animal models of allergic contact dermatitis, topical pimecrolimus was found to be effective. In human studies of allergic contact dermatitis pimecrolimus demonstrated significantly more efficacy than the control treatment. As well, the effectiveness of pimecrolimus 0.6% cream was comparable to 0.1% betamethasone-17-valerate; however, pimecrolimus was not associated with any of the side effects characteristic of a topical steroid. Topical application of pimecrolimus is not associated with skin atrophy. Pimecrolimus is effective and safe in both children and adults with atopic dermatitis. When pimecrolimus 1% cream has been applied to adult atopics, improvement has been observed as early as the first week, with a 72% reduction in severity after 3 weeks. Pharmacokinetic studies have shown very low blood levels of pimecrolimus following topical application, with no accumulation after repeated applications. Following application of pimecrolimus cream occasional transient irritation may be experienced at the application site. Similar results have also been found in children aged 3 months and older following application of pimecrolimus 1% cream. Topical pimecrolimus in psoriasis appears to exhibit a dose-dependent therapeutic effect under semi-occlusive conditions. Pimecrolimus has an enormous potential as a new treatment of inflammatory skin diseases. It has been shown to be effective in atopic and allergic contact dermatitis, with a favorable adverse-effects profile, which includes little effect on the systemic immune response.

Key words: pimecrolimus, SDZ ASM 981

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Introduction

Pimecrolimus (SDZ ASM 981) is one of the new class of novel ascomycin immunomodulating macrolactams, and was developed for the treatment of inflammatory skin diseases. Ascomycin, first isolated as a fermentation product of *Streptomyces hygroscopicus* var. *ascomycetus* in the early 1960s,[1] was initially researched primarily for its antifungal properties. However, it was more than 20 years later that ascomycin was investigated for its structural and immunomodulatory

properties.[1] Two major derivatives were shown to be topically effective in treating inflammatory skin diseases: SDZ ASM 981 and SDZ 281-240. Pimecrolimus (SDZ ASM 981) is currently the most advanced ascomycin macrolactam under development. Pimecrolimus is a colourless, solid compound[2] with a molecular weight of 810.48 daltons.[2]

Interest in pimecrolimus has been intensive because of its significant anti-inflammatory activity and immunomodulatory capabilities and its low systemic immunosuppressive potential.

Mechanism of action

The mechanism of action of pimecrolimus is the blockage of T cell activation. Ascomycin macrolactams are immunophilin ligands that bind to a specific cytosolic receptor. Pimecrolimus binds to immunophilin macrophilin-12, also known as FK506 binding protein, and FKBP-12. Tacrolimus (FK506) and rapamycin also bind to macrophilin-12.[2] Like tacrolimus and cyclosporin A,[3] the mechanism of action of pimecrolimus involves its binding to macrophilin-12.[2] The pimecrolimus-macrophilin complex then binds to the cytosolic enzyme calcineurin phosphatase.[4] Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase that regulates the translocation of cytosolic components of nuclear factors, which in turn regulate the promoter activities of several mediators during mRNA transcription.[4] By inhibiting the action of calcineurin, the pimecrolimus-macrophilin complex prevents the dephosphorylation of the cytoplasmic component of the nuclear factor of activated T cells (NF-AT).[1] NF-AT regulates the mRNA transcription of a number of inflammatory cytokines; therefore, pimecrolimus blocks this transcription, especially Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) type cytokines.[4] Other cytokines, including IL-5, IL-10 and TFNα, are decreased in production by pimecrolimus in a dose-dependent manner.[4]

Pimecrolimus also targets mast cells which play an important role to anti-inflammatory activities.[5] Pimecrolimus inhibits not only the transcription and synthesis of cytokines from mast cells, but also the release of preformed mediators serotonin and β-hexosaminidase[1] by the inhibition of Fcε-RI-mediated degranulation and secretion.[2] It is important to note that all the inhibition processes occur only when pimecrolimus is bound to macrophilin-12.[2] It is of interest that, during a study of murine mast cell line CPII,[2] it was found that pimecrolimus did not inhibit the transcription of a reporter gene which was under the control of the human TFNα promoter in the murine dendritic cell line, and had no effect on IL-8 release from keratinocytes, fibroblasts and endothelial cells. This is an indication of the specificity of the pharmacologic activity of pimecrolimus.[2]

The first study of the gene expression analysis of blood cells was performed on seven patients with psoriasis, who had been treated with oral pimecrolimus 30 mg twice daily.[6] Blood samples were taken from the patients prior to treatment and after 13 or 14 days of treatment. Gene chips were used for gene expression analysis and 7129 genes were surveyed. Kehren *et al.*[6] found a genomic profile of pimecrolimus of approximately 100 genes. As well, it was demonstrated that pimecrolimus treatment caused a strong down-regulation of the expression of mRNA for genes associated with the macrolactam target pathway and inflammation. However, no changes were found in the mRNA for genes which generally reflect drug related side effects, like those associated with apoptosis, stress induction and enzymatic induction. Therefore, Kehren *et al.*[6] concluded that the genomic analysis of blood cells from psoriatic patients treated with pimecrolimus supports the specific anti-inflammatory nature of the therapy.

Studies

Animal Models

Animal models have been used to evaluate the effectiveness of both topical and systemic pimecrolimus. Meingassner *et al.*[7,8] performed several studies in mouse, rat and pig models with allergic contact dermatitis, in which topical pimecrolimus displayed a very high level of effectiveness. In the pig model a statistically significant anti-inflammatory effect was observed at concentrations as low as 0.04%.

The atrophogenic effects of clobetasol-17-propionate 0.05%, a potent topical corticosteroid, and pimecrolimus 0.3% topical formulation on pig skin (which has similar qualities to human skin) were compared. Corticosteroids are known to cause skin atrophy after repeated topical or systemic use, affecting both the dermis and epidermis. Meingassner *et al.*[7] applied the corticosteroid and pimecrolimus formulations for 13 days under the same conditions. Pimecrolimus had no effect on skin texture or thickness, which suggests that it lacks an atrophogenic ability while displaying an effectiveness similar to potent corticosteroids in treatment of allergic contact dermatitis.

Oral therapy with cyclosporine A, tacrolimus and pimecrolimus for allergic contact dermatitis has been evaluated in mouse and rat models.[7,8] In the mouse model, pimecrolimus was found to be as effective as cyclosporine A following oral ingestion and slightly superior after subcutaneous administration.[7] As well, it was found that pimecrolimus at doses up to 4×90 mg/kg does not impair sensitization, unlike cyclosporine A or tacrolimus at doses of 4×60 mg/kg and 4×30 mg/kg, respectively.[8] In addition, pimecrolimus contrasts cyclosporine A and tacrolimus by inhibiting ongoing secondary inflammatory response, but not impairing the primary immune response in allergic contact dermatitis.[8]

Systemic immune reactions were also studied by Meingassner *et al.*[7] in two rat models, a graft-versus-host reaction and an allogenic kidney transplantation. In the graft-versus-host model the right hind foot pad of the rat was injected subcutaneously with spleen cells from allogenic rats. Pimecrolimus was administered by subcutaneous injection the same day as the

spleen cell injection, and 1, 2, and 3 days later. Pimecrolimus was inactive at low subcutaneous doses of 0.1 and 0.3 mg/kg, and had only a minimal effect at higher doses of 3 and 9 mg/kg. In the allogenic kidney transplantation model, following the transplant of the kidney from the donor rat to the recipient rat, pimecrolimus of 20% concentration in solid dispersion was administered daily to the recipient for the first 14 days after transplant. It was found that pimecrolimus prevented organ rejection at oral doses of 15.6 mg/kg and higher. Cyclosporin A was effective at dosages three times lower than this. Meingassner *et al.*[7] concluded that pimecrolimus, unlike cyclosporine A, has a large therapeutic window, within which treatment of skin inflammation is possible with no adverse effects on the immune system.

Neckermann *et al.*[9] performed a study to examine the effectiveness of systemic and topical pimecrolimus on hypomagnesaemic hairless rats compared with its vehicle. The magnesium deficiency in rats produces a pruritic rash, which resembles the clinical features of atopic dermatitis. The oral administration of pimecrolimus was in the form of a solid solution of 20% active drug. Daily doses of 4.0 or 12.5 mg/kg body weight were administered on three consecutive days by gavage (5 mL/kg body weight) following the development of clinical signs. Treatment with pimecrolimus 12.5 mg/kg cleared up the eruption, with pronounced reduction within the first day following the start of therapy. Complete inhibition was observed within 4 days; however, signs of recurrence appeared 2 days after the last dose and continued to be present until the end of the study period.[9] The vehicle-treated rats demonstrated no changes in the extent of pruritus or skin lesions.

Neckermann *et al.*[9] also evaluated the utility of pimecrolimus as a prophylactic agent using hypomagnesaemic hairless rats. Seven daily doses of either pimecrolimus 12.5 mg/kg body weight or vehicle were administered beginning from day 3 of the diet until day 9. The use of pimecrolimus using the prophylactic regimen almost completely suppressed the onset of the erythematous pruritic eruption with only one of seven pimecrolimus-treated rats developing slight erythema on day 9.[9] None of the rats treated in a prophylactic manner with systemic pimecrolimus exhibited any signs of pruritus during the study period; however, all vehicle treated rats developed severe erythematous lesions.

The topical administration of pimecrolimus to hypomagnesaemic hairless rats was conducted by dissolving the pimecrolimus 0.4% in ethanol/propylene glycol.[9] Topical treatment was applied to one ear and vehicle to the other. The ear treated with the active drug displayed significant reduction in erythematous swelling within one day of the start of therapy with the inflammation being suppressed for 3 days after the last dose; however, recurrence was noted 4 or 5 days after therapy had been discontinued. The use of topical pimecrolimus in a prophylactic manner was also effective with suppression of the inflammation of the ears of five rats treated. It is important to note that topical treatment of the ear with pimecrolimus did not affect the erythematous lesions on the trunk, and the degree of pruritus did not differ between the active drug and vehicle treated groups. Neckermann *et al.*[9] indicate that topical pimecrolimus at the concentration used in this study did not appear to have a systemic effect. Both the therapeutic and prophylactic treatment of the rats resulted in an inhibition of histamine levels; however, when there was clinical evidence of erythema, histamine blood levels in the pimecrolimus treated animals were similar to the vehicle treated rats.

Human Studies

Atopic dermatitis (Table 1 and Table 2)

There have been several human studies that have evaluated the efficacy of pimecrolimus in atopic dermatitis. Van Leent *et al.*[10] studied the topical application of 1% pimecrolimus twice daily versus once daily for 3 weeks on two comparative target areas (one on the left arm, one on the right arm), and compared efficacy with a placebo cream in 34 adult patients. Patients treated twice daily displayed significant improvement as early as 2 days of starting treatment, and within 3 weeks there was a mean reduction of 71.9% in the severity of atopic dermatitis. The median time for partial clearance of the disease at the treated sites was 8 days. By the end of the treatment period, 12 of 16 patients achieved partial clearance and three patients were totally clear.[10] The efficacy in those patients treated once daily was less than in the group receiving twice daily applications. In the once daily group, the mean reduction of severity of atopic dermatitis was 37.7% and none of the patients reached complete clearance by the end of the study period; only three of 18 patients achieved partial clearance.[10] The 1% pimecrolimus cream was significantly more effective than the placebo cream, with no skin irritation or any local adverse effects observed. After the completion of therapy, symptoms of atopic dermatitis returned gradually, without rapid rebound.

Laboratory studies were performed on all the patients with no relevant changes being observed. The concentration of pimecrolimus in whole blood was measured and only two of 129 samples exceeded the limit of quantification (0.1 ng/mL). The authors suggest that it is safe to assume that these two samples were contaminated, as they were taken from separate patients on two separate occasions.[10] Van Leent *et al.*[10] concluded that 1% pimecrolimus cream applied twice daily to the body surface is an effective and safe treatment of atopic dermatitis, with a dose dependent trend.

Luger *et al.*[11] assessed the use of topical pimecrolimus in patients with atopic dermatitis who had 5% to 30% total body surface area involvement ($n = 260$). Subjects were randomly assigned treatments of pimecrolimus cream 0.05%, 0.2%, 0.6%, or 1.0%, vehicle cream or 0.1% betamethasone-17-valerate cream. The 0.1% betamethasone-17-valerate cream is a potent corticosteroid, which was used as an internal control. The

Table 1 Use of topical pimecrolimus on adult patients with atopic dermatitis

Author	Study Type	Participants	Treatment Regime	Efficacy	Pimecrolimus Blood Level Concentrations	Safety
Van Leent *et al.*[10]	randomized, double blind, placebo controlled, right-and-left comparison study	34 adult patients daily vs. placebo cream for	pimecrolimus 1% cream applied twice daily or once 3 weeks on two comparative target areas (one on the left arm, the other on the right arm	patients with partial clearance ($0 < ADSI \leq 2$): 75% (pimecrolimus twice daily), 13% (placebo twice daily), 17% (pimecrolimus once daily), and 0% (placebo once daily; patients with complete clearance ($ADSI = 0$): 19% (pimecrolimus twice daily), and 0% (all other treatments)	only two of 121 blood samples were above the limit of quantification (0.1 ng/mL) – authors suspect the two samples were the result of contamination	no clinically significant adverse effects observed
Luger *et al.*[11]	double-blind, randomized, parallel-group, multicentre dose-finding study	260 adult patients (ages 18 years or older)	four concentrations of pimecrolimus cream (0.05%, 0.2%, 0.6%, 1.0%), vehicle cream, or 0.1% betamethasone-17-valerate (BMV) cream applied twice daily to affected areas for 3 weeks	patients with moderately clear or better (>50% improvement): 88.1% (BMV), 16.3% (vehicle), 53.3% (1.0% cream), 54.8% (0.6% cream), 32.6% (0.2% cream), and 0.05% cream failed to show significant therapeutic effect	systemic exposure was consistently low with 72% of measurements below limit of quantification	mild to moderate application site reactions were the most common adverse events reported, with the majority of reactions beginning on the first day and resolving within 3 days; next most common adverse events were pruritus and worsening of atopic dermatitis
Van Leent *et al.*[12]	two non-controlled, open-label, multiple topical dose study	total 52 adult patients (ages 18 years or older)	pimecrolimus 1% cream applied twice daily for 3 weeks, followed by twice daily application on an 'as-needed' basis for up to 1 year	not reported	blood concentration levels lower than limit of quantification (0.5 ng/mL) at 3 weeks: 78%; individual maximum concentrations ranged from < 0.5 to 1.4 ng/mL, with a single isolated value at 4.6 ng/mL – authors suspect this sample was contaminated blood concentration levels lower than limit of quantification (0.5 ng/mL) over 1 year: 98%; maximum concentration seen over the 1 year period was 0.8 ng/mL	well tolerated both locally and systemically

Table 2 Use of topical pimecrolimus on pediatric patients with atopic dermatitis

Author	Study Type	Participants	Treatment Regime	Efficacy	Pimecrolimus Blood Level Concentrations	Safety
Kapp *et al.*[12]	multicentre, parallel group, double-blind, controlled study	251 pediatric patients (ages 3–23 months)	pimecrolimus 1% cream or vehicle applied twice daily according to need for 1 year; emollients and medium-high potency topical corticosteroids were allowed to be used for flares not controlled by study medication	patients without incidence of flares at 6 months: 70.1% (pimecrolimus 0.1%), 32.6% (vehicle with emollients and corticosteroid use)	not reported	no significant difference in the incidence of adverse events between the two treatment groups
Harper *et al.*[13]	four open-label pharmaco-kinetic studies	total of 58 paediatric patients (ages 3 months to 14 years)	pimecrolimus 1% cream applied twice daily for 3 weeks; 11 patients continued treatment over 1 year on an 'as-needed' basis	not reported	blood concentration levels lower than 2 ng/mL: 93%; blood concentration levels lower than 5 ng/mL: 60%	no evidence of accumulation over time; no systemic side effect observed
Morris *et al.* In Paller[14]		pediatric patients (ages 5–16 years)	pimecrolimus 1% cream applied twice daily for 3 weeks	mean reduction in dermatitis: 70%	blood concentration levels below limit of detection (0.4 ng/mL): 60%	transient mild to moderate warmth or burning
Harper *et al.*[15]	open and non-controlled study	10 pediatric patients (ages 1–4 years) started	pimecrolimus 1% cream applied twice daily for 3 weeks	average EASI decreased by 12.8, with a range of improvement of 8–89% from baseline score at the end of treatment period	blood concentration levels lower than 0.5% ng/mL: 63%; individual maximum concentrations ranging from < 0.5 to 1.8 ng/mL	no serious adverse events occurred
De Prost *et al.*[25]	multicentre, double-blind study	713 pediatric patients (ages 2–17 years)	pimecrolimus 1% cream or current standard of care, including use of emollients (SoC) applied twice daily for 12 months; medium-high potency topical corticosteroids were allowed if flares occurred	patients without incidence of flares at 6 months: 61% (pimecrolimus 1%), and 34% (SoC); patients without incidence of flares at 12 months: 51% (pimecrolimus 1%), 28% (SoC); proportion of patients who did no use corticosteroids within 12 months: 57% (pimecrolimus 1%), and 32% SoC	not reported	systemic immune response not affected
Wahn *et al.*[26]	multicentre, controlled, double-blind study	713 pediatric patients (ages 2–17 years)	pimecrolimus 1% cream or vehicle applied twice daily for 1 year	patients without incidence of flares at 6 months: 61% (pimecrolimus 1%), and 34.2% (vehicle); patients without incidence of flares at 12 months: 50.8% (pimecrolimus 1%), and 29.3% (vehicle); patients treated with pimecrolimus had a longer time to first flare than vehicle and significantly reduced the use of corticosteroids	not reported	mild and transient feelings of warmth/ burning were reported by 10.5% of pimecrolimus treated patients

Table 2 *Continued*

Author	Study Type	Participants	Treatment Regime	Efficacy	Pimecrolimus Blood Level Concentrations	Safety
Whalley *et al.*[28]	two randomized, double-blind clinical trials	total of 403 pediatric patients (ages 2–17 years)	pimecrolimus 1% cream or vehicle applied for 6 weeks	significant improvement in quality of life scores in both treatment groups, however pimecrolimus treated group showed significantly greater improvements over vehicle treated group	not reported	not reported
Boguniewicz *et al.*[29]	two randomized, multicentre studies, followed by an open-label study	total of 403 pediatric patients (ages 2–17 years)	pimecrolimus 1% cream or vehicle applied twice daily for 6 weeks, followed by twice daily application on an 'as-needed' basis for 20 weeks	patients with clearance or almost clearance (IGA score = 1 or 0): 34.8% (pimecrolimus 1%), and 18.4% (vehicle)	not reported	most common reports of mild to moderate application site burning, which resolved early in treatment (18.1%)
Wahn *et al.*[30]	non-controlled, open-label, multiple topical dose, multicenter study	20 pediatric patients (ages 3–23 months)	pimecrolimus 1% cream or vehicle applied twice daily or 3 weeks	median reduction of EASI from baseline to day 22: −78%; marked eduction of EASI was observed as early as day 4	blood concentration levels lower than limit of quantification (0.1 ng/mL): 31%; blood concentration levels lower than 0.5 ng/mL: 71%; individual maximum concentrations ranged from < 0.1 to 2.29 ng/mL	adverse application site reactions reported, however the reactions were considered not to affect the well-being of the patients
Ho *et al.*[31]	double-blind, multicenter, vehicle controlled study, followed by an open label extension	186 pediatric patients (ages 3–23 months)	pimecrolimus 1% cream or vehicle applied twice daily for up to 6 weeks, followed by continuation of treatment of pimecrolimus patients twice daily for 20 weeks	patients with clearance or almost clearance (IGA score = 1 or 0) at 6 weeks: 54.5% (pimecrolimus 1%), and 23.8% (vehicle); mean reduction of EASI: −61.78% (pimecrolimus 1%), and +7.25% (vehicle); patients with no or minimal pruritus: 72.4% (pimecrolimus 1%), and 33.3% (vehicle)	not reported	well tolerated throughout study and extension

assigned treatment was applied twice daily for 3 weeks to all affected areas, except for the face. The greatest efficacy was observed with betamethasone-17-valerate over all other treatments. Pimecrolimus 1.0% and 0.6% cream were both effective treatments; pimecrolimus 0.05% treatment had no significant therapeutic effect, which was expected. All treatments helped to improve the pruritus. By the end of the study period signs of atopic dermatitis appeared to be moderately clear or better (> 50% improvement) with pimecrolimus 1.0% (53.3%) and 0.6% creams (54.8%) compared with only 16.3% improvement with the vehicle.[11]

Very few systemic adverse effects were observed and none were considered to be related to the treatment.[11] Local reactions at the application site included burning, warmth, stinging, smarting, pain and soreness. These occurred most frequently with pimecrolimus 1% cream, were of mild to moderate severity, and most reactions were transient, beginning on day 1 of treatment and resolving within the first 3 days. Luger *et al.*[11] concluded that topical application of pimecrolimus was well tolerated and effective in treating atopic dermatitis, displaying a dose-response trend, with 1.0% cream being the most effective concentration of pimecrolimus. It is possible that a treatment period exceeding 3 weeks would have resulted in a greater therapeutic effect.[11]

Topical pimecrolimus has been found to be safe and effective in children. Studies have been performed in children as young as 3 months. Kapp *et al.*[12] conducted a 6 month double-blind randomized long term study on safety and efficacy of pimecrolimus in infants aged 3–23 months with atopic eczema ($n = 251$ patients). Pimecrolimus, used as an early intervention treatment, was compared with a group that received a current standard of care for atopic eczema, that is, a regimen of emollients, and corticosteroids. Vehicle cream was used instead of pimecrolimus in the control group in order to maintain the study blind. Medium to high potency corticosteroids were used to treat flares not controlled by pimecrolimus. Following the use of corticosteroids, pimecrolimus was resumed. The primary efficacy parameter was the incidence of flares during the 6 month study period.[12] It was found that pimecrolimus provided better control of atopic dermatitis than standard emollient treatment, with 70.1% of the pimecrolimus patients completing the treatment period without any flares compared with 32.6% in the control group. The mean number of days of corticosteroid use was about twice as great in the standard emollient treatment group than the pimecrolimus group. Therefore, pimecrolimus significantly reduced the incidence of flares and the dependence on corticosteroids in infants with atopic dermatitis as young as 3 months.

Harper *et al.*[13] performed short- and long-term pharmokinetic studies in children aged 3 months to 14 years with moderate to severe eczema ($n = 58$ patients). Initial treatment involved the application of pimecrolimus cream 1% twice daily for 3 weeks. Two studies followed 11 patients over 1 year who continued treatment on an 'as needed' basis. Blood samples were take throughout the studies. Patients as young as 3 months had blood levels, which were consistently low; 93% of pimecrolimus blood concentrations were lower than 2 ng/mL and 60% of samples were lower than 0.5 ng/mL. This pattern is similar to those found in adults.[13] The authors concluded that pimecrolimus was well tolerated in the treatment of pediatric patients, even as young as 3 months, regardless of extent of body surface involved, of lesions or of duration of treatment.[13]

Systemic absorption is very low and no accumulation is observed. A European study of 5–16-year-old children treated twice daily for 3 weeks with pimecrolimus 1% cream demonstrated a 70% mean reduction in dermatitis by the end of the treatment period.[14] The dermatitis recurred following the discontinuation of the pimecrolimus therapy. A pediatric study of ten patients, 1–14 years old, with moderate to severe atopic dermatitis on 23% to 69% of their body surface area, was performed for 3 weeks.[15] The patients were treated twice daily with pimecrolimus 1% cream for 3 weeks. By the end of the treatment period there was an improvement of Eczema Area and Severity Index (EASI) by 8% to 89% from the baseline score with the seven patients who completed the treatment.[15] No serious adverse events were reported and no clinically relevant adverse-effects were observed upon physical examination, vital signs or laboratory safety parameters. A total of 63 blood samples were taken throughout the treatment period; 63% of those samples had pimecrolimus concentrations less than 0.5 ng/mL, with the maximum concentration ranging from less than 0.5 to 1.8 ng/mL. The highest pimecrolimus blood levels were approximately 20 times lower than levels associated with no toxicity in animal toxicity studies and a human study where oral pimecrolimus was administered.[15] Blood samples drawn at the end of the study period demonstrated no accumulation of pimecrolimus after several weeks of treatment. While orally administered pimecrolimus may degrade into several minor metabolites, the metabolism of topically applied pimecrolimus is negligible through the skin;[15] therefore systemic exposure to pimecrolimus due to topical application is probably negligible.

Psoriasis

There has been one study of the treatment of psoriasis with pimecrolimus. Whereas the atopic dermatitis studies used a cream formulation of pimecrolimus, an oral formulation was used to treat psoriasis. Rappersberger *et al.*[16,17] evaluated the safety, tolerability and efficacy of treatment of patients with moderate to severe chronic plaque psoriasis by comparing five dose levels of oral pimecrolimus (5 mg o.d., 10 mg o.d., 20 mg o.d., 20 mg b.i.d. and 30 mg b.i.d.) to a placebo. Thirty-eight patients were treated with pimecrolimus and 10 patients treated with the placebo for 4 weeks. All five dose levels of pimecrolimus were well tolerated, with no serious adverse

effects; the only frequent adverse effect noted was a mild to moderate, transient feeling of warmth when the treatment was applied. As well, no clinical changes were noted with any of the physical and biochemical examinations. Pimecrolimus doses of 20 mg b.i.d and 30 mg b.i.d. were observed to have the greatest reduction in the Psoriasis Area and Severity Index (PASI) of 60% and 75%, respectively, compared to 4% for placebo.[16]

Topical treatment of psoriasis using pimecrolimus is usually restricted to mild disease because of its limited effectiveness,[18] due to the thick scaling and limited penetration into lesional psoriatic skin.[19] Pimecrolimus 0.3%, 1.0% ointment, ointment base and clobetasol-17-propionate were compared over 2 weeks in ten adult patients with stable chronic plaque-type psoriasis.[18] The treatments were applied daily for 2 weeks under occlusion using Finn chambers. Pimecrolimus 0.3% cream had only a mild effect on psoriatic lesions up to day 10, followed by little further resolution. Initially, 1% pimecrolimus provided a weaker response compared with clobetasol-17-propionate; however, by the end of the treatment period there was no significant difference between the two treatments in the ability to clear lesions. No adverse events were reported throughout the treatment period. Therefore, pimecrolimus, when applied under occlusion, was found to be effective in clearing psoriatic plaques in a dose dependent manner.

Mrowietz *et al.*[20] performed a study to evaluate the effectiveness of pimecrolimus without occlusion in 23 adults with plaque-like psoriasis. Pimecrolimus 1% cream was compared with vehicle, 0.005% calcipotriol ointment and 0.05% clobetasol-17-propionate ointment. The study medications were applied to the test sites twice daily for 21 days. Erythema, induration and scaling were evaluated for therapeutic effect. Pimecrolimus was significantly more effective than the vehicle, with improvement in scores of 50.0% and 28.6%, respectively in the two groups. However, both calcipotriol and clobetasol had a greater effectiveness than pimecrolimus, with improvements of 71.4% and 87.5%, respectively. This is the first study to report significant therapeutic effect by pimecrolimus in treating psoriasis without occlusion, where pimecrolimus had greater efficacy than the vehicle, although less efficacious than calcipotriol and clobetasol ointment.[20]

Allergic contact dermatitis

The effectiveness of topical anti-inflammatory drugs have often been tested on experimentally-established allergic contact dermatitis.[1] In a study by Queille-Roussel *et al.*[21] the effectiveness of two different formulations of pimecrolimus 0.2% and 0.6% cream, vehicle, and betamethasone-17-valerate 0.1% cream was compared in 66 adults with nickel contact dermatitis. The patients were treated twice daily for up to 12 days. Both formulations of the pimecrolimus were significantly more effective than the vehicle. As well, pimecrolimus 0.6% creams were comparable with betamethasone-17-valerate 0.1% cream. There were no serious side effects observed with pimecrolimus cream. This treatment of nickel allergic contact dermatitis with pimecrolimus is the first controlled trial where a topical non-corticosteroid has demonstrated efficacy.

Safety and tolerability of topically applied pimecrolimus

Topical application of pimecrolimus appears to be safe when used in both children and adults. The most common adverse events expected are application site reactions, for example, burning, feeling of warmth, smarting, pain, and soreness.[11] Most application site reactions have been found to be of mild to moderate severity. To some extent, subjects applying the vehicle have also reported these reactions. In patients applying pimecrolimus 1% cream the applications site reactions appear to be transient, usually beginning on the first day of treatment and resolving within the first 3 days of therapy.[11]

An important advantage of topical pimecrolimus over the topically applied corticosteroids is that the ascomycin derivative does not induce skin atrophy when applied to normal skin.[22] The traditional treatments of inflammatory skin diseases have been potent topical steroids. However, long term use of these treatments is limited due to several adverse events, including skin atrophy. Topical corticosteroids are known to inhibit collagen synthesis in the skin, leading to skin atrophy.[23,24] Queille-Roussel *et al.*[22] conducted a comparison study of pimecrolimus 1% cream, its vehicle, betamethasone-17-valerate 0.1% cream and triamcinolone acetonide 0.1% cream in 16 healthy adult volunteers. Each treatment was applied to the volar aspect of the forearms twice daily, 6 days a week, for 4 weeks. By using ultrasound it was determined that there was no relative change to the total skin thickness of the pimecrolimus treated sites compared with the vehicle, even by the last examination. However, application of topical corticosteroids resulted in significant reduction in skin thickness, which was apparent as early as day 8. None of the patients reported any adverse events at the application sites. This study demonstrated a clear lack of atrophogenic potential of pimecrolimus 1% cream.[22]

De Prost *et al.*[25] performed a study of 713 children, ages 2–17 years with atopic eczema, comparing the use of pimecrolimus 1% cream with a standard of care (SoC) regimen, which included the use of emollient creams and medium-high potency topical corticosteroids, for long term management of atopic eczema in children, each applied twice daily for up to 12 months. Topical corticosteroids were used only if flares occurred; following corticosteroid use, the assigned treatment was resumed. It was found that the pimecrolimus significantly reduced the use of corticosteroids; 57% of the pimecrolimus treated patients did not use corticosteroids within 12 months, while only 32% of the SoC group avoided corticosteroid use.[25] As well, pimecrolimus significantly reduced the incidence of flares over the 6 and 12 month periods. Within 6 months, 61% of pimecrolimus patients were without flares, however only

34% of the SoC group had none. At month 12, 51% of pimecrolimus patients had no flares while only 38% of emollient patients were free from flares.[25] Therefore, the use of pimecrolimus significantly reduced both the amount of time before flares first occur and the total number of flares, along with the frequency of topical corticosteroid use.[25–27] Use of pimecrolimus improves the quality of life of the patient.[28] Clinical trials by Whalley *et al.*[28] of 403 pediatric patients compared pimecrolimus 1% cream with its vehicle over 6 weeks. Significantly greater improvements were associated with the pimecrolimus treatment than the vehicle.[28]

Topically applied pimecrolimus has been associated with low systemic absorption. For example, in a study by Harper *et al.*[15] children aged 1–4 years with atopic dermatitis were treated twice daily for 3 weeks with pimecrolimus 1% cream. The blood concentrations of pimecrolimus were consistently low even in the patients with the most extensive surface areas treated (up to 69% body surface area). Furthermore, pimecrolimus did not accumulate over the treatment period and no systemic effects were detected. Similar findings have been reported by other investigators,[29,30] even with patients as young as 3 months,[31] and also in adult patients.[32,33]

Comparison with tacrolimus

Tacrolimus (FK 506) is also a newly developed immunomodulator that is being used for treatment of atopic dermatitis and several other inflammatory skin disorders. Tacrolimus was discovered from the fermentation broth of the soil microbe *Streptomyces tsukuba* found in Japan.[34] Initially, tacrolimus was used systemically to prevent the rejection of new grafts in patients who had undergone allograft transplants. The mechanism of action of tacrolimus and pimecrolimus is similar. Both tacrolimus and pimecrolimus bind specifically to the immunophilin macrophilin-12, which blocks the action of the phosphatase calcineurin. This ultimately results in the suppression of gene transcription and responsiveness of T cells.[35] Tacrolimus has a molecular weight of 822 daltons and is absorbed readily through damaged skin barrier. The patient's skin absorbs lower quantities of tacrolimus as lesions heal, which helps reduce adverse effects.[36]

While the structures of pimecrolimus and tacrolimus are similar, the structure of pimecrolimus possesses two different chemical group attachments; pimecrolimus is 20 times more lipophilic than tacrolimus.[37] A higher lipophilicity allows pimecrolimus to have a higher affinity to the skin; as a result, pimecrolimus has a lower permeation potential through the skin, with a skin-selective pharmacologic profile.[38] As well, although the mechanism of pimecrolimus and tacrolimus is similar, their selectivity is different. Meingassner *et al.*[39] compared pimecrolimus to both cyclosporine A and tacrolimus, demonstrating that pimecrolimus may have a weaker immunosuppressing capacity. Bochelen *et al.*[40] demonstrated that pimecrolimus has about a 3-fold lower inhibition potential of calcineurin than tacrolimus. This may result in pimecrolimus being less effective at lower doses but may be as effective as tacrolimus at higher doses.[40] In the United States, tacrolimus is indicated for the treatment of moderate to severe atopic dermatitis in individuals aged 2 years and higher; pimecrolimus is indicated for treatment of mild to moderate disease in the same age group.

According to Stuetz *et al.*[38] pimecrolimus may need to be administered in significantly higher amounts than cyclosporine A or tacrolimus to prevent organ rejection in animal models. Meingassner *et al.*[42] support this statement with rat models of allogeneic kidney transplants. The lowest oral dose of pimecrolimus, which prolonged the survival of the animal to 100 days or longer, was 15 mg/kg. In comparison, 5 mg/kg of cyclosporine A and 1 mg/kg of tacrolimus were required to achieve the same long-term survival. Although pimecrolimus appears to have lower immunosuppressive properties, this may in turn allow pimecrolimus to have a more selective immunomodulatory activity than the other two treatments, as well as a lower potential for systemic immunosuppression when administered orally than tacrolimus.[42] Animal models have demonstrated that treatments of systemically applied pimecrolimus does not cause toxic adverse effects, like nephrotoxicity, hepatotoxicity or hypertension.[5]

Conclusion

Pimecrolimus has enormous potential as a topical treatment for inflammatory skin disease. It is highly efficient in blocking T cell activation and inhibiting the synthesis of inflammatory cytokines. Pimecrolimus is effective in dermatoses such as atopic dermatitis and allergic contact dermatitis, and is indicated in the United States for the short-term and intermittent long-term therapy of mild to moderate atopic dermatitis in non-immunocompromised patients aged 2 years and older where alternative conventional therapies are deemed inadvisable because of potential risks, or for patients who are not adequately responsive to, or are intolerant of conventional therapies. Adverse effects experienced with topical application have been transient events, generally of mild to moderate severity. Unlike topical corticosteroids, the ascomycin is not associated with the development of skin atrophy. This is an advantage compared to topical corticosteroids, particularly when considering long-term use and application at certain anatomic sites such as the face, neck and genital areas. Pimecrolimus has demonstrated a low blood level concentration, even over long term treatment periods with a low potential for affecting the systemic immune response when applied topically. The significant anti-inflammatory activity, immunomodulatory capabilities and highly favourable adverse effects profile of pimecrolimus make it an ideal treatment for several inflammatory skin diseases.

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